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Effects of *Hypericum perforatum* (St. John's wort) on the metabolism and uptake of γ-aminobutyric acid (GABA)

by
Richelle Booker ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Psychiatry

Edmonton, Alberta
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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Effects of *Hypericum perforatum* (St. John's wort) on the metabolism and uptake of γ-aminobutyric acid (GABA) submitted by Richelle Booker in partial fulfillment of the requirements for the degree Master of Science in Psychiatry.

DEDICATION

For Dad, Mom and Rea

how lucky I have been to share the ups and downs of this adventure we call life with three incredible, warm, kind, funny, and loving people like you

ABSTRACT

The therapeutic herbal preparation St. John's wort (SJW), derived from *Hypericum perforatum*, has gained widespread public recognition as popular alternative to the traditional pharmacological management of mild and moderate depression. The anecdotal claims of efficacy have been validated, to a degree, by an abundance of clinical trial evidence. A great deal of pre-clinical research has been conducted to date, including *in vitro* and *in vivo* research, although the mechanism of action remains unclear. A significant goal of research is to determine the active constituent(s) responsible for the therapeutic effects of SJW.

GABA has an established link to anxiety disorders and has been implicated in the neurochemistry of depression. The interaction of SJW with GABA metabolism and/or uptake may provide an explanation for the antidepressant and anxiolytic activity of the herb.

SJW was found to inhibit the activity of GABAse, a bacterial preparation of GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), although none of the individual constituents tested were responsible for the inhibition. The

inhibitory effects of SJW on GABAse activity were further investigated, using an HPLC procedure, in order to determine which of the two enzymes, GABA-T or SSADH, accounted for the observed inhibition of GABAse. The results indicated that SJW is an inhibitor of GABA-T activity. SJW was also found to dose-dependently inhibit the uptake of GABA by rat cortical prisms. A variety of constituents were tested (hypericin, hyperforin, quercetin, quercitrin, isoquercitrin, pseudohypericin, and kaempferol) and of these, only hyperforin demonstrated significant activity. The inhibition with hyperforin was evident at concentrations at which it is normally present in SJW. A number of commercially available SJW preparations were tested for GABA uptake inhibition and a range of inhibitory activity was observed, emphasizing the importance of determining the hyperforin concentrations of SJW preparations in the future.

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List of Abbreviations

AOAA	Aminooxyacetic acid
ANOVA	Analysis of variance
$CaCl_2$	Calcium chloride
Cl^-	Chloride ion
CSF	Cerebrospinal fluid
CVT	CV Technologies
CYP	Cytochrome P450
DA	Dopamine
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th edition
EDTA	Ethylenediaminetetraacetate
EEG	Electroencephalogram
Erk	Extracellular signal regulated protein kinase
GABA	γ-Aminobutyric acid
GABA-T	GABA-α-oxoglutarate transaminase; GABA-transaminase
GAD	GABA decarboxylase
GLU	Glutamate
GSH	Glutathione
HCl	Hydrochloric acid

HPLC	High performance liquid chromatography
5-HT	5-Hydroxytryptamine; serotonin
HYPC	Hypericin
HYPF	Hyperforin
IC_{50}	Inhibitory concentration 50%
ID	Internal diameter
IM	Incubation medium
KCl	Potassium chloride
α-KETO	α-Ketoglutarate
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDD	Major depressive disorder
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
NA	Noradrenaline
Na^+	Sodium ion
NaCl	Sodium chloride
NADP	β-Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium pyrophosphate
NaOH	Sodium hydroxide
NPY	Neuropeptide Y
OD	Optical density

ODS	Octadecylsilane
OPA	o-Phthalaldehyde
PLP	Pyridoxal-5-phosphate
PLZ	Phenelzine
RPM	Rotations per minute
SA	Succinic acid
SSA	Succinic semialdehyde
SSADH	Succinic semialdehyde dehydrogenase
SEM	Standard error of the mean
SJW	St. John's wort
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TCP	Tranylcypromine
THF	Tetrahydrofuran
TRIS	Tris buffer
UV	Ultraviolet
WHO	World Health Organization

1. INTRODUCTION

The antidepressant mechanism of action of St. John's wort (SJW) remains unknown, despite the widespread use of the herb. There have been numerous clinical trials conducted and St. John's wort has reported efficacy as not only an antidepressant, but also as an anxiolytic. In this thesis, the effects of St. John's wort on GABAergic mechanisms were examined.

1.1 Depression

Depression is one of the most common psychiatric conditions, with approximately 5 – 6% of the population depressed at any given time (Potter and Hollister, 2001). The characteristics of depression include significant negative changes in mood, accompanied by a variety of symptoms that persist and interfere with the ability to function in normal day-to-day activities. Symptoms include sleep disturbances, inability to concentrate, thoughts of death and dying, thoughts of suicide, weight gain or loss, irritability, loss of interest in activities and loss of pleasure. These symptoms are generally present for a period of at least two weeks, and occur on more days than they are absent [Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV)].

1.1.1 Subtypes of depression

Depression is heterogeneous in presentation and in etiology (Arana and Rosenbaum, 2000). The DSM-IV is often used to assist the diagnosis process. Subtypes of depression include major depressive disorder (MDD), dysthymic disorder, the depressed phase in bipolar disorder, atypical depression, hostile and irritable subtypes, depression associated with grief and bereavement, and depression with psychotic features. MDD is associated with major depressive episodes while dysthymia involves milder MDD symptoms that have persisted for at least 2 years (Parikh et al., 2001). Atypical depression involves symptoms that are not normal features of depression, such as mood reactivity, a significant increase in weight or appetite, excess sleep, and leaden paralysis (Parikh et al., 2001). Anxious depression (involves significant symptoms of anxiety), atypical depression, melancholia (involves symptoms such as loss of interest in almost all activities and lack of reactivity to usually pleasurable stimuli), psychotic depression (involves symptoms such as delusions or hallucinations), post-partum depression (generally occurs within 4 weeks following the birth of a child) and seasonal affective disorder (involves recurrent episodes of depression at characteristic times of the year) are all considered to be subtypes of major depressive disorder (Parikh et al., 2001).

1.1.2 Major depressive disorder

MDD is relatively prevalent and is generally recurrent, or chronic, in nature. A World Health Organization (WHO) report indicated that levels of disability among primary care patients were greater in patients with MDD than those with other chronic conditions, including hypertension, diabetes mellitus, arthritis and low back pain (Parikh et al., 2001). The full recovery rate associated with the use of antidepressant drugs is approximately 50%, and between 10 and 15% of depressed patients will not improve (Arana and Rosenbaum, 2000; Parikh et al., 2001). The response rates are lower in patients diagnosed with co-morbid conditions, and in such patients it is often necessary to try a combination of different medications.

1.1.3 Comorbidity

Major depression is often accompanied by anxiety, and the symptoms of anxiety are frequently responsive to antidepressant treatment (Brier et al., 1985; Arana and Rosenbaum, 2000; Friede et al., 2001). The selective serotonin reuptake inhibitors (SSRIs) are useful in the treatment of depression associated with panic disorder, while the benzodiazepines are useful in treating symptoms of anxiety. Imipramine, a tricyclic antidepressant (TCA) has been used with success in the treatment of generalized anxiety disorder (Sheehan et

al., 1980; Zitrin et al., 1983). The SSRIs and venlafaxine are increasingly being used for the treatment of generalized anxiety disorder (Arana and Rosenbaum 2000; Westenberg, 1996).

Other co-morbid conditions include depression complicating borderline personality disorder and depression co-morbid with substance abuse.

1.1.4 Refractory depression

There are a number of patients who respond only partially, or not at all, to antidepressant therapy. There are three major recommendations suggested for treating refractory depression, and these include optimizing the dose of medication, combining medications to augment the individual effects of each other, and substituting medications with others of the same class, or from another class, of antidepressant (Arana and Rosenbaum, 2000).

1.2 Neurochemistry of depression

1.2.1 Monoaminergic neurotransmission

Noradrenaline (NA) is active throughout the brain and is released from neurons originating in the locus ceruleus. Serotonin (5-hydroxytryptamine; 5-HT) is released from neurons originating in the

raphé nuclei of the brainstem. These neurotransmitters are involved in arousal, vigilance, attention, mood, sensory processing, and appetitive functions (Arana and Rosenbaum 2000).

Following release from the nerve terminal, excess 5-HT and NA is removed from the synapse, terminating further interaction with the postsynaptic membrane, via an active reuptake process. The transporters responsible for the uptake of 5-HT and NA belong to a family of Na^+/Cl^- dependent transporters and are found on neurons and glia (Neary et al., 2001). Following reuptake, the neurotransmitter is either taken into vesicles for future release or degraded by monoamine oxidase (MAO).

1.2.2 Biogenic amine theory

While the original biogenic amine hypothesis of depression suggested that depression was caused by a NA deficiency at central synapses (Schildkraut, 1965), it is now believed that a functional deficit of both NA and 5-HT is involved. Evidence to support the biogenic amine theory of depression came from experiments where drugs capable of restoring synaptic levels of both 5-HT and NA were found to exhibit antidepressant activity (Lapin and Oxenkrug, 1969). This hypothesis was further reinforced by the revelation that reserpine,

a drug that depletes neurons of NA and 5-HT, induces symptoms of depression in animals and humans (Klein, 1968). Most of the antidepressant drugs currently in use [such as MAO inhibitors (MAOIs), TCAs, SSRIs and venlafaxine] increase the functional availability of NA and/or 5-HT (Hyman et al., 1995).

1.2.3 Discrepancies in the biogenic amine theory

There has been some controversy surrounding the biogenic amine theory of depression, and modifications of the original proposal were necessary. A significant departure from the simplistic deficiency theory relates to the delay in therapeutic onset in spite of immediate neurochemical effects. While the biochemical effects of the drugs occur rapidly, it may take several weeks before the symptoms of depression remit (Oswald et al., 1972; Quitkin et al., 1986). A temporal relationship between drug administration and clinical response should exist if the biochemical changes, such as increased synaptic levels of 5-HT and NA, are the sole mechanism of antidepressant action. Further inconsistencies have been found, such as the discovery that cocaine, a drug known to increase synaptic levels of NA, is not effective in the treatment of depression. The theory is further contradicted by the discovery of the 'novel' antidepressants, such as

iprindole, mianserin, bupropion and viloxazine, that behave as neither inhibitors of MAO activity or 5-HT/NA uptake (Sulser et al., 1978).

1.2.4 Modified biogenic amine theory

The biogenic amine theory has been modified and will continue to evolve as the complex interactions of different neurochemical systems are elucidated. Changes in the regulation of postsynaptic receptors are now believed to contribute to the neurochemistry of depression, as reductions in the density of β-adrenergic and/or 5-HT_2 receptors have been observed following chronic treatment with the TCAs, MAOIs, and some of the novel antidepressants (Charney et al., 1981; Enna et al., 1981; Baker et al., 1989; Bourin and Baker, 1996).

1.2.5 GABA and anxiety and depression

The role of GABA in the etiology of anxiety disorders has been well established, and the benzodiazepine anxiolytics act by enhancing GABAergic transmission (Martin, 1987). The mechanism of at least some of the antipanic drugs, such as alprazolam and phenelzine, is believed to involve GABA as well (Bourin et al., 1988). An association between GABA and depression was suggested in clinical trials where levels of GABA in cerebrospinal fluid (CSF) of depressed patients were found to be lower than those of healthy patients (Gold et al., 1980;

Gerner and Hare, 1981; Kasa et al., 1982). It has also been reported that patients with unipolar affective disorders, bipolar disorder, or secondary depression have lower plasma GABA levels than matched controls (Petty et al., 1993; Petty, 1994).

Post-mortem binding studies conducted on depressed patients have revealed a reduced density of $GABA_A$ binding sites, an effect associated with chronic antidepressant administration (Suzdak and Gianutsos, 1985). However, it has also been reported in animal studies that the density and affinity of such binding sites are unaltered by antidepressant administration (Lloyd et al., 1989).

Antidepressant effects of the GABA agonists progabide, baclofen, muscimol, and fengabine have been observed in both animal models and in human subjects (Lloyd et al., 1989), although this subject remains controversial (Paykel et al., 1991). An upregulation of $GABA_B$ receptors in rat cortex has been reported following chronic administration of several types of antidepressants and repeated electroconvulsive therapy (Lloyd et al., 1989; Gray and Green, 1987). However, this effect has also been disputed (McManus and Greenshaw 1991; Cross and Horton, 1987).

1.2.6 GABA neurotransmission

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system, with approximately 30 to 50% of all neurons involved in GABAergic transmission (Petty et al., 1993). GABA is found in millimolar concentrations in many regions of the brain, with the highest levels in the substantia nigra and the globus pallidus (Perry, 1982).

1.2.7 Metabolism of GABA

The metabolism of GABA is highly regulated and dependent on the availability of GABA precursors (Westergaard et al., 1995). The GABA shunt, shown in Figure 1 (page 11), illustrates the metabolic pathway of GABA and the link to the Kreb's cycle. Glutamate (GLU) is decarboxylated by glutamic acid decarboxylase (GAD) to form GABA. Pyridoxal-5-phosphate (PLP), the main co-enzymatic form of pyridoxine, acts as a cofactor in the reaction. GAD is found only in GABAergic neurons. GABA-α-oxoglutarate transaminase (GABA-transaminase; GABA-T) is the enzyme responsible for the degradation of GABA. In contrast to GAD, GABA-T is found in regions of the brain not containing GABAergic neurons (DeLorey and Olsen, 1994). GABA-T is also a PLP-dependent enzyme, but GAD has a lower affinity for PLP than does GABA-T.

1.2. 8 GABA uptake and enzymatic degradation

The activity of excess GABA at a synapse is terminated by reuptake into either the presynaptic nerve terminal or surrounding glial cells. GABA reuptake is mediated by a specific transporter system, and the transport is energy-temperature- and ion-dependent. The driving force for reuptake comes from the movement of Na^+ down its concentration gradient. GABA that is taken up into the nerve terminal may be re-packaged into vesicles for reuse, while GABA taken up by glial cells will be metabolized. Glial cells lack GAD and are therefore not capable of synthesizing GABA (DeLorey and Olsen, 1994). In glial cells GABA-T catalyzes the reaction in which GABA is converted to succinic semialdehyde (SSA) and glutamate (GLU) and α-ketoglutarate is transaminated. SSA is not detected as a metabolite of GABA *in vivo* because succinic semialdehyde dehydrogenase (SSADH) is very efficient in oxidizing SSA, which subsequently re-enters the Kreb's cycle as succinic acid (SA). The enzymatic degradation of GABA, as well the reuptake process, are both pharmacological targets of a number of drugs.

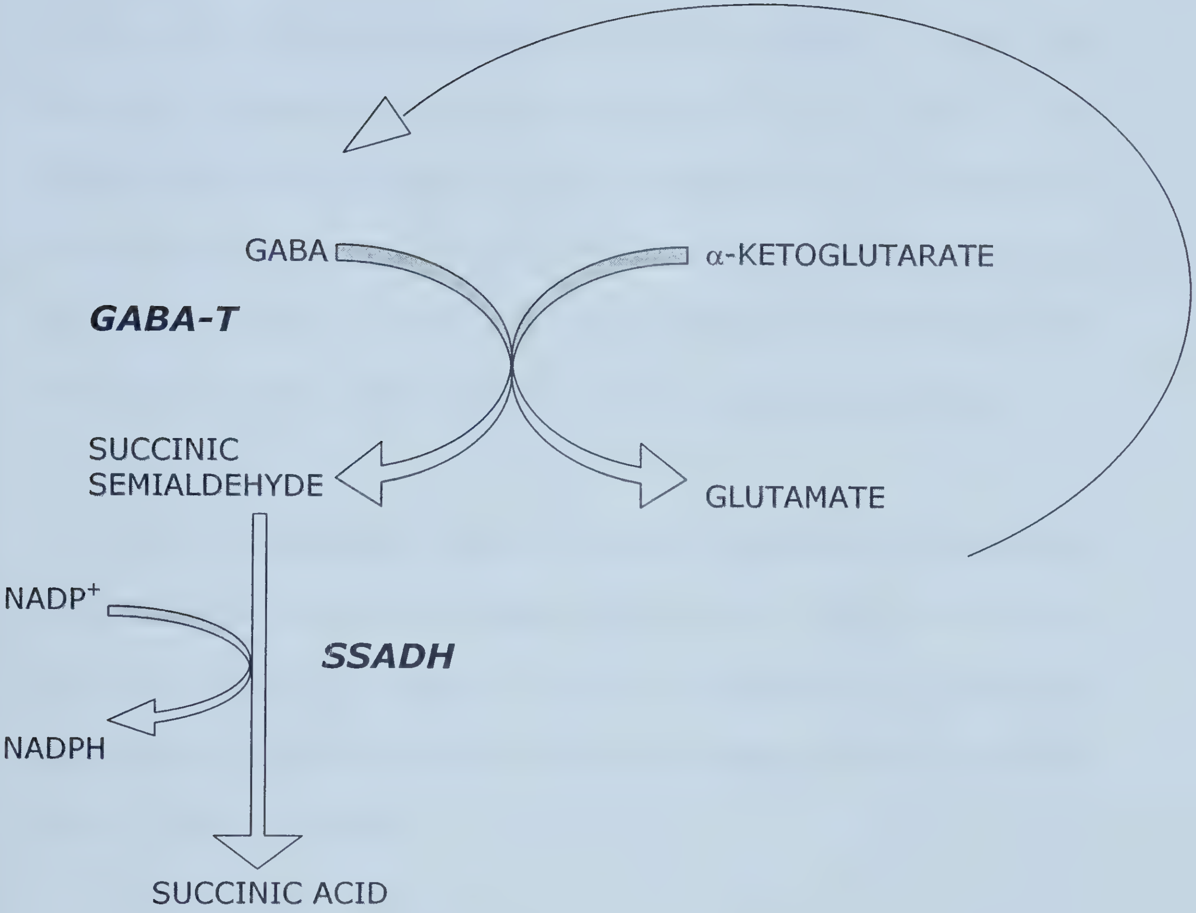


Figure 1. The GABA shunt. GABA is metabolized by GABA-transaminase (GABA-T) to succinic semialdehyde (SSA) and glutamate. SSA is metabolized to succinic acid by succinic semialdehyde dehydrogenase (SSADH), which may then enter the Kreb's cycle. NADP$^+$ is oxidized to NADPH during the SSADH reaction.

1.2.9 GABAergic drugs

The triazolobenzodiazepines alprazolam and adinazolam, which are agonists at the benzodiazepine site on the $GABA_A$ receptor, have reported antidepressant efficacy (Rickels et al., 1985). The antidepressant and antipanic drug phenelzine (PLZ), in addition to being an inhibitor of MAO, also inhibits GABA-T, which leads to a marked elevation of brain GABA levels (Popov and Matthies, 1969; Baker et al., 1991; Todd and Baker, 1995; Baker et al., 2000).

Several drugs that inhibit the uptake or catabolism of GABA or act as agonists at the $GABA_A$ receptor are useful clinically as anticonvulsants (e.g. tiagabine, vigabatrin, clonazepam). These drugs are reported to have both anxiolytic and antipanic efficacy (see Baker et al., 2000 for review).

1.3 Treatment of depression

The treatment of depressive disorders may involve psychotherapy, including cognitive behavioral therapy, interpersonal psychotherapy, brief dynamic psychotherapy, supportive psychotherapy, and cognitive-behavioral analytic systems of psychotherapy or pharmacotherapy, or it may involve a combination of

both psychotherapy and pharmacotherapy (Reesal et al., 2001). The details of pharmacotherapy will be addressed below.

1.3.1 Antidepressant drugs

The manner in which antidepressant drugs exert their therapeutic influence remains somewhat unclear. The rationale behind the development of the antidepressants, in general, relates to the proposal that depression is caused by a functional deficiency of certain neurotransmitters. In particular, antidepressant drugs were developed to enhance monoaminergic transmission by increasing the synaptic availability of NA and/or 5-HT (Schildkraut, 1965; Arana and Rosenbaum, 2000). The biogenic amine hypothesis of depression has formed the foundation upon which the majority of experimental models have been designed, and antidepressant pharmacotherapy has been targeted to correct these proposed deficiencies, although it now seems likely that other neuroactive neurochemicals such as amino acids, steroids and neurotrophic factors are involved (Young, 2002; Le Melledo and Baker, 2002). However, the antidepressants currently available are classified as exerting their primary influence on the metabolism, reuptake, or selective receptor antagonism of 5-HT, NA, or both (Potter and Hollister, 2001).

1.3.2 MAOIs

The MAOIs were accidentally introduced in the 1950s when an anti-tuberculosis drug was observed to induce antidepressant and stimulant characteristics in patients (Arana and Rosenbaum, 2000). The drug, iproniazid, was found to inhibit MAO activity. MAO is an enzyme that is found in most tissues of the human body, with the exception of red blood cells and blood plasma (Blaschko, 1952). The enzyme is found in the outer membrane of mitochondria, where it takes part in the oxidative deamination of a range of monoamines (Fowler and Ross, 1984). The enzyme exists in two isozymes, MAO-A or MAO-B. MAO-A and MAO-B are differentiated based on substrate preference and selective inhibition (Johnson, 1968), and are the products of two different genes (Bach et al., 1988; Hsu et al., 1988). MAO-A preferentially metabolizes NA and 5-HT, while MAO-B preferentially metabolizes β-phenylethylamine. Clorgyline is a selective inhibitor of MAO-A, while MAO-B is inhibited by (-)-deprenyl (Johnson, 1968). Through the use of autoradiography, MAO-A has been identified in the locus ceruleus, substantia nigra, paraventricular thalamus, raphé nuclei, solitary tract nucleus, inferior olives, interpeduncular nucleus, claustrum, and peripheral tissues such as the liver, vas deferens, heart, superior cervical ganglia and the pancreas (Saura Marti et al., 1990). MAO-B has been found in the ependyma,

raphé nuclei, paraventricular thalamus, posterior pituitary, platelets and liver (Saura Marti et al., 1990).

There are three inhibitors of MAO that have been developed for clinical use in psychiatry as antidepressants. Phenelzine (PLZ) and tranylcypromine (TCP) are irreversible inhibitors of MAO-A and MAO-B and moclobemide is a reversible inhibitor of MAO-A. Selegiline, used in the treatment of Parkinson's disease, is a selective inhibitor of MAO-B.

The major adverse effects associated with the use of MAOIs include postural hypotension, insomnia, agitation, sexual dysfunction, weight gain, nausea and vomiting (Wyatt et al., 1971; Rabkin et al., 1984; Murphy et al., 1985). PLZ and TCP, through irreversible inhibition of MAO-A, can also cause an increase in blood pressure when given at the same time as foods containing vasoactive amines such as tyramine (see below). The use of MAOIs clinically in the pharmacological treatment of depression and Parkinson's disease has advanced with the discovery of novel reversible and/or selective compounds. (-)-Deprenyl, an irreversible and selective MAO-B inhibitor, although not particularly successful as an antidepressant, is now used in the treatment of Parkinson's disease (Strolin Benedetti

and Dostert, 1992). The reversible MAO-A inhibitor moclobemide is used in the treatment of depression (Da Prada et al., 1990).

Although rare, the most serious adverse effect associated with the use of MAOIs is the 'cheese effect.' This effect, also referred to as the 'tyramine pressor effect,' includes symptoms such as headache, nausea, fever and a potentially lethal hypertension. The syndrome may be precipitated or exacerbated by the concomitant ingestion of foods rich in sympathomimetic amines such as tyramine. Tyramine can be found in foods such as aged cheese, red wine, chocolate, smoked meats, and yeast products. Inactivated intestinal MAO-A allows unmetabolized tyramine to subsequently reach the circulation where it exerts an indirect pressor effect by releasing NA from storage vesicles. The released NA can then initiate an exaggerated activation of α_1-adrenergic receptors, leading to a drastic elevation of blood pressure (Sandler, 1981). Reversible inhibitors of MAO-A are much less likely than irreversible inhibitors to initiate such a severe reaction (Da Prada et al., 1989; Nair et al., 1993).

1.3.3 TCAs

The TCAs were developed in the 1950s when analogues of the antipsychotic drug chlorpromazine were being tested. The TCAs were

named after the characteristic three-ring structure. Imipramine is the prototypical TCA, while later tricyclics varied in either the central ring or the side chain (Lader, 1980). These drugs are generally absorbed rapidly following administration and are metabolized extensively (Rudorfer and Potter, 1989). The TCAs are generally highly protein-bound in the plasma. Four major routes, including desmethylation of a side chain, N-oxidation of a side chain, hydroxylation of the ring structure, at various positions, and glucuronidation are involved in the metabolism of TCAs (Rudorfer and Potter, 1989; Potter and Manji, 1990; Young, 1991).

The adverse effects associated with the use of TCAs are mediated primarily through antagonism of peripheral and central muscarinic and α-adrenergic receptors. The anticholinergic effects include dry mouth, blurred vision, constipation, urinary retention, and excessive sweating, while the adrenergic effects include hypotension, tachycardia, arrythmias, and postural hypotension. Cardiac toxicity associated with TCA use is primarily due to quinidine-like effects, resulting in slowing of intracardiac conduction. The use of TCAs should be avoided in patients with cardiac conditions such as left bundle branch block or prolonged QT interval. TCAs may cause erectile dysfunction in men (Arana and Rosenbaum, 2000). The emergence of

side effects may have an impact on patient compliance, and may necessitate a change in antidepressant.

An overdose with a TCA may lead to death resulting from drug-induced cardiac arrythmias, hypotension, or uncontrollable seizures (Arana and Rosenbaum, 2000). The immediate symptoms of overdose include antimuscarinic effects such as dry mucous membranes, warm dry skin, mydriasis, blurred vision, reduced bowel motility, and urinary retention. These physical symptoms may be followed by central nervous system depression, ranging from drowsiness to coma, and respiratory arrest.

1.3.4 Selective serotonin reuptake inhibitors (SSRIs)

In the 1970s, fluoxetine was shown to cause selective inhibition of 5-HT (serotonin) uptake in rat synaptosomes (Arana and Rosenbaum, 2000). Fluoxetine was the first drug of this class, which now includes fluvoxamine, sertraline, paroxetine and citalopram. The SSRIs are generally better tolerated than the MAOIs and TCAs, as they produce fewer side effects. The major adverse effects include nausea, headache and sexual dysfunction. This class of drug also exhibits a

much lower risk for lethal overdose compared to the TCAs and MAOIs (Arana and Rosenbaum, 2000).

1.3.5 Some other commonly used antidepressants

Bupropion is a phenelthylamine compound used in the treatment of depression. The drug bears some structural resemblance to amphetamine and to the sympathomimetic diethylpropion (Arana and Rosenbaum, 2000), but its effects on uptake of DA and NA are rather weak (Stahl, 2000). In general, the drug is well tolerated, although patients may develop symptoms of agitation, restlessness, insomnia, anxiety or gastrointestinal upset. In comparison to some of the older antidepressants, bupropion lacks anticholinergic side effects, is devoid of cardiac effects and is associated with a low incidence of sexual dysfunction (Golden and Nicholas, 2000).

Venlafaxine acts as an inhibitor of NA and 5-HT uptake at higher doses, while selectively inhibiting 5-HT at lower doses. The drug is free of anticholinergic and antihistaminergic effects and the adverse effects associated with venlafaxine use include anxiety, nervousness, nausea, insomnia, sedation, dizziness and constipation (Beauclair et al., 2000).

Nefazodone is a phenylpiperazine compound that weakly inhibits 5-HT uptake, but primarily acts via blockade of postsynaptic 5-HT_2 receptors. The most frequent side effects associated with nefazodone include headache, dry mouth, and nausea. Nefazodone can be used to treat depression-associated insomnia, and in contrast to some of the other newer antidepressants, nefazodone does not cause sexual dysfunction (Garlow et al., 2000).

Mirtazapine is tetracyclic in structure and acts as a α_2-adrenergic, 5-HT_{2A} and 5-HT_{2C} receptor antagonist. The drug also blocks 5-HT_3 and histamine receptors, but does not affect 5-HT or NA reuptake. Mirtazapine is reported to have a faster onset of action compared to the SSRIs. The drug is associated with minimal anticholinergic effects, although due to a sedating effect, bedtime use is recommended (Stahl, 2000).

Trazodone is a triazolopyridine derivative that bears no chemical relation to the MAOIs or TCAs. The drug weakly inhibits the reuptake of 5-HT, while acting as an antagonist at postsynaptic 5-HT_2 receptors. A major metabolite, *m*-chlorophenylpiperazine, is proposed to be a postsynaptic 5-HT receptor agonist. Trazodone may cause sedation

and postural hypotension due to α-adrenergic receptor blockade (Garlow and Nemeroff, 2000).

1.4 Herbal products

The use of herbal products has exploded over the past few years, and sales figures reach into the billions of dollars ever year as consumers turn towards an alternative approach to medicine. A 1997 survey conducted in the United States revealed that the percent of patients using herbal medicine has increased 380% since 1990 (Eisenberg et al., 1998). There is a belief among the general public that herbal products are safer alternatives than prescription and conventional medications (Bray et al., 2002). As the trend towards holistic medicine continues, psychiatric patients are seeking natural remedies to treat a number of conditions, including depression, anxiety, and nervousness. The 'Prozac of the plant kingdom', St. John's wort (SJW) has become popular as a natural antidepressant. In Europe, SJW sales exceeded $6 billion in one year, and the herbal remedy outsold fluoxetine four-fold as the leading treatment for depression (DiCarlo et al., 2001). In 1997, the sale of SJW in the United States topped $48 million, and over the period of one year, sales increased by 2800% (Barnes et al., 2001).

A significant concern regarding the use of traditional herbal medicine involves the lack of control and regulation surrounding the manufacture, production and sale of such products. At the present time, herbal medications are classified as food and dietary supplements and are exempt from the mandatory safety and efficacy standards of prescription and over-the-counter pharmaceuticals (Marwick, 1995). The burden of conventional research is often neglected with herbal products, as manufacturers are discouraged by the difficulty in obtaining patents for herbal products (Ang-Lee et al., 2001). There are reports that the potency of herbal medications vary from manufacturer to manufacturer, that plants are misidentified, that products are contaminated with pesticides, and that some products contain toxins or prescription drugs (Ang-Lee et al., 2001). While some manufacturers have made efforts to standardize their products, the benefits of doing so are uncertain, as it is thought that some products may achieve efficacy through combined or synergistic effects (Ang-Lee et al., 2001). Though standardized, some preparations have been found to vary considerably standard in potency (Ang-Lee et al., 2001). One specific consequence resulting from the lack of herbal regulations is the inconsistency among products. This factor was investigated in the research described and will be discussed in further detail below.

1.5 SJW

1.5.1 Origins of SJW

SJW preparations are composed of the leaves and flowering tops of *Hypericum perforatum* (DiCarlo et al., 2001). The perennial weed has five-petalled yellow flowers. The name 'Hypericum' is Greek; '*hyper*' meaning 'above,' '*eikon*' meaning 'image' (DiCarlo et al., 2001). It has been reported that, in ancient times, Greeks and Romans placed SJW branches above images and statues, and believed that the plant had mystical powers that would protect them from evil spirits. The second part of the name, *perforatum*, describes the tiny translucent excretory glands on the leaves that resemble perforations (DiCarlo et al., 2001; Barnes et al., 2001). The more common name, St. John's wort, was chosen as the plant blossoms around the 24th of June, St. John's Day. When the leaves of the plant are crushed, a red pigment is released and this pigment is said to have symbolized the spilling of St. John's blood when he was beheaded. The term '*wort*' is old English for 'plant' (DiCarlo et al., 2001).

1.5.2 Historical and modern use of SJW

SJW has been utilized for several centuries to treat an array of medical conditions. In the middle Ages, SJW was reportedly used in 'magic potions' prepared to protect humans and animals from 'witches,

demons, and evil disease.' SJW was later used as a treatment for scrapes, bruises, infections, nervousness, melancholia, and headache (Orth et al., 1999; Poldinger 2000; Rezvani et al., 1999).

The use of SJW in modern medicine is also varied, and the plant has reported efficacy as an antibacterial, antiviral, anti-neoplastic, anti-inflammatory and anxiolytic (Barnes et al., 2001). SJW was shown to be active against gram-positive bacteria, and demonstrated significant potency against methicillin-resistant *Staphylococcus aureus* (Reichling et al., 2001). In cancer patients, the antineoplastic efficacy of the herb has been demonstrated. Two of the constituents are used in photodynamic therapy as photosensitizers. The photosensitizing agent is selectively taken up into, and retained by, malignant cells and these specific cells are then irradiated. The combination of the radiation with the photosensitizer initiates both apoptotic and necrotic cell death, in a targeted manner (Agostinis et al., 2002; Schempp et al., 2002). The most popular use of SJW, however, is as an alternative antidepressant, in the treatment of mild to moderate depression (Barnes et al., 2001).

1.5.3 Geographical Distribution of SJW

SJW is one species, out of more than 370, belonging to the *Hypericum* genus (Nangia et al., 2000; Whiskey et al., 2001). The plant can be found growing in temperate climates worldwide and is regarded as a common weed in the United States, Canada, Europe and Australia. The composition of the plant varies greatly among species, and ecological factors, such as climate, environment, time of harvest, and processing and extraction procedures all contribute to the chemistry of the medicinal product (Muller et al., 1998; Narstedt and Butterweck, 1997;).

1.5.4 Constituents of SJW

SJW contains several potentially active constituents, belonging to a number of different classes, such as the anthraquinones, the flavonoids, the phloroglucinols, phenols, and volatile oils. Each class, and the constituents identified thus far, is described below.

The anthraquinone constituents, also referred to as naphthodianthrones, include hypericin (HYPC), pseudohypericin, isohypericin and cyclopseudohypericin. The total hypericin content, which includes both hypericin and pseudohypericin, is

between 0.1 and 0.3%. The flavonoid constituents include flavonols

(such as kaempferol and quercetin), flavones and glycosides (such as hyperoside, isoquercitrin and quercitrin), biflavonoids (including biapigenin and amentoflavone) and catechins. Phloroglucinol constituents include hyperforin (HYPF) and adhyperforin, which are found in concentrations of 2 to 4.5%, and 0.2 to 1.9%, respectively. Caffeic, chlorogenic, *p*-coumaric, ferulic, hydroxybenzoic and vanillic acids have also been found to be constituents of SJW. The volatile oil constituents include methyl-2-octane, n-nonane, methyl-2 decane, n-undecane, pinene, terpineol, geraniol, myrcene, limonene, carophyllene and humulene (Barnes et al., 2001; Whiskey et al., 2001).

1.5.5 Pharmacokinetics

In pharmacokinetic studies conducted with a SJW preparation standardized to 0.3% hypericins, HYPC reached peak plasma levels in approximately four hours after a 900 to 1800mg dose of SJW, and plasma concentrations were found in the nM to μM range (Barnes et al., 2001; Wonnemann et al., 2001). The elimination of HYPC is slow, with a half-life of 2 hours. HYPF levels peak approximately 3.5 hours following administration of a SJW dose of between 900 and 1800mg and plasma concentrations are in the nM range (Biber et al., 1998). The half-life of HYPF is 9 hours. HYPC and HYPF reach steady-state

after approximately three to four days of dosing, and the plasma concentrations are comparable to those attained with other antidepressants (Biber et al., 1998). In healthy volunteers, the administration of 300mg SJW tablets, containing approximately 14.8mg of HYPF, resulted in plasma concentrations of 150ng/mL (DiCarlo et al., 2001).

1.6 SJW as an antidepressant

1.6.1 Clinical trial evidence

Although the antidepressant mechanism of action remains unclear, the therapeutic efficacy of SJW extract has been confirmed in a vast array of clinical trials (Bauer et al., 2001; Kennedy et al., 2001; Whiskey et al., 2001). A large body of clinical trial evidence has shown that SJW has antidepressant efficacy superior to placebo in the treatment of mild to moderate depression (Nathan 2001). In clinical trials comparing SJW with traditional antidepressants, SJW was found to have equivalent antidepressant activity. The trials included comparisons with the TCAs (including imipramine, desipramine and amitriptyline), maprotiline and the SSRIs fluoxetine and sertraline (Behnke et al., 2002; Brenner et al., 2000; Vorbach et al., 1994; Whiskey et al., 2001).

A recent meta-analysis, involving 2291 patients, compared the use of SJW to standard antidepressants, and revealed that overall SJW preparations were significantly superior to placebo and equal in efficacy to the TCAs (Linde et al., 2000). The average duration of the trials included in the study was between four and six weeks and patients were diagnosed using DSM-IV criteria.

In a trial evaluating the antidepressant efficacy of SJW in pediatric patients it was found that 300mg of SJW daily was a viable pharmacological option for the treatment of mild to moderate depression (Hubner and Kirste, 2001).

However, in a recently published double-blind, randomized, and placebo-controlled trial conducted on patients diagnosed with MDD it was concluded that SJW was not effective in the treatment of major depression (Shelton et al., 2001). In addition, the results of a recent clinical trial, conducted by the *Hypericum* Depression Trial Study Group (2002), revealed that SJW is no more effective than placebo in the treatment of major depression. The randomized, double-blind, placebo-controlled trial involved 12 academic and community psychiatric research clinics in the US. The adult outpatients were diagnosed with major depressive disorder, and assigned to receive

either placebo, SJW, or sertraline (an SSRI antidepressant) over a period of 8 weeks. At the conclusion of the trial, it was concluded that neither sertraline nor SJW produced results different than those produced by placebo (*Hypericum* Depression Trial Study Group).

A major criticism of many of the clinical trials has been that there is little consistency in the brand of product tested, and as such, meta-analyses are of little use as there is large inter-trial variability. The quantity of constituents has been shown to be inconsistent between SJW preparations. Among extracts of SJW, there may be significant variation in the concentration of constituents, and the correlation between pharmacological activity and certain constituents indicates that the medicinal benefit derived from selected extracts will also vary considerably (Sloley et al., 2000). A comparison of German St. John's wort preparations revealed a significant discrepancy between the reported quantity of active ingredient on the label to what was actually found in the preparation (Wurglics et al., 2001). The quantity of HYPF and HYPC was measured in each of the preparations and there was a large variation between the different brands. HYPF content, in the average daily dose, ranged from not quantifiable in one product to 35mg in another (Wurglics et al., 2001). A double-blind, multicentre trial evaluated the antidepressant efficacy of two SJW

preparations which varied in HYPF content, and a larger response was observed in patients that received the extract containing 5% HYPF, compared to patients receiving the extract containing 0.5% HYPF (Barnes et al., 2001). The use of different brands of SJW among the clinical trials makes it difficult to compare results, and it has been recommended that a standardized SJW preparation be used in future clinical trials.

1.6.2 Adverse effects and drug interactions

The lack of adverse effects associated with SJW use has contributed largely to the popularity of this herb as an antidepressant. The rate of side effects was reported to be ten times lower than the rate associated with synthetic antidepressant use (Schulz, 2001; Whiskey et al., 2001). In a clinical trial involving 3,250 patients, the overall incidence of side effects was 2.4% (Jensen et al., 2001). The most frequently reported complaints involved gastric irritation (0.6%), allergic reactions (0.5%), fatigue (0.4%) and restlessness. Photosensitivity has been reported, but only when doses exceed the recommended 900 to 1800mg of SJW, daily.

It was initially believed that the use of SJW was not associated with any particular negative health consequences, aside from the few

minor side effects described above, and the Physician's Desk Reference for Herbal Medicine initially reported that there were no risks of herb-drug interactions (Whiskey et al., 2001). However, with the widespread use of SJW, in conjunction with a number of prescription medications, it has become quite clear that there are indeed a number of risks associated with co-administered medications. The co-administration of SJW and SSRI antidepressants has been associated with the emergence of serotonin syndrome and is not recommended. This syndrome arises as a consequence of excess 5-HT activity, and includes symptoms such as flushing, sweating, ataxia, muscle twitching and confusion (Ang-Lee, 2001). In addition to the interaction between serotonergic agents and SJW, there are a number of pharmacokinetic interactions that have been identified.

The cytochrome P450 (CYP) enzymes are responsible for the metabolism of a vast array of exogenous and endogenous substrates. In addition to the substrates, there are a number of agents that act as either inducers or inhibitors of the enzymes, which further complicates the metabolism of a number of drugs. SJW has been identified as both an inducer and inhibitor of the CYP enzymes and has been implicated in an assortment of significant drug interactions. SJW was found to inhibit the activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4

(Obach, 2000; Barnes et al., 2001). When the crude SJW extract was fractioned and tested, the flavonoid constituent biapigenin was a potent, competitive inhibitor of CYP3A4, CYP2C9 and CYP1A2 (Obach, 2000). HYPF inhibited CYP2D6 activity in a non-competitive manner (Barnes et al., 2001; Obach et al., 2000). In a study using human hepatocytes, SJW and HYPF were both shown to induce CYP 3A4 mRNA expression (Bray et al., 2002; Barnes et al., 2001; Durr et al., 2000), although short-term administration of SJW had no effect on CYP activity overall (Wang et al., 2001). HYPF is also a potent ligand for the pregnane X receptor, a nuclear receptor involved in the regulation of CYP3A4 expression (Barnes et al., 2001). The most frequent interactions between SJW and traditional medications are listed in Table 1.

A human study involving healthy volunteers showed that SJW treatment, over a two-week period, lead to a 1.4 fold increase in P-glycoprotein levels (Bray et al., 2002). In an alternate study, P-glycoprotein expression, in peripheral blood lymphocytes, was increased more than 4 times the baseline expression in healthy volunteers treated with SJW (Hennessey et al., 2002). P-glycoprotein,

Table 1. Drug interactions induced by SJW.

CO-MEDICATION	RESULTS OF INTERACTION	POSSIBLE MECHANISM
Cyclosporine	Lowering of blood cyclosporine levels; rejection of transplant	Hepatic enzyme induction; induction of intestinal P-glycoprotein
Oral contraceptives	Breakthrough bleeding	Hepatic enzyme induction
Theophylline	Lowering of plasma theophylline levels	Hepatic enzyme induction
Warfarin	Lowering of plasma warfarin levels; decreased anticoagulant effect	Hepatic enzyme induction
Amitriptyline	Lowering of plasma amitriptyline levels	Hepatic enzyme induction
Indinavir	Lowering of plasma indinavir levels	Hepatic enzyme induction; induction of intestinal P-glycoprotein
Digoxin	Lowering of plasma digoxin levels	Induction of intestinal P-glycoprotein
Nefazodone	Serotonin syndrome	Synergistic serotonin uptake inhibition
Sertraline	Serotonin syndrome	Synergistic serotonin uptake inhibition
Paroxetine	Lethargy or incoherence	Synergistic serotonin uptake inhibition

(Adapted from Nathan, 2001).

also referred to as the multi-drug resistance gene, is a transporter that actively pumps drugs out of the cell. Some drugs are inhibitors of the pump, while some are inducers. The induction of P-glycoprotein by SJW will affect drugs that are pumped by this transporter, such as cyclosporine and indinavir (Bray et al., 2002; Hennessey et al., 2002).

1.6.3 Animal studies

There are a number of animal models that can be used to predict the antidepressant and anxiolytic efficacy of drugs in humans. The evidence from such animal studies indicates that SJW extract has properties that resemble those of clinically established antidepressant drugs (Franklin and Cowen, 2001; Jensen et al., 2001).

The antidepressant effects of SJW have been evaluated in the acute form of escape deficit induced by unavoidable stress, a chronic model of escape deficit and a model of anhedonia (based on the finding that repeated stressors prevent the development of appetitive behavior). The administration of SJW was found to acutely protect animals from the effects of unavoidable stress, to reverse the chronic escape deficit state that had been caused by exposure to repeated stressors, and to preserve the capacity of the animal to acquire motivated behaviors (Gambarana et al., 2001).

The forced swimming test is a well-established animal model that is useful for predicting the antidepressant activity of drugs in humans (Bourin et al., 1996; Porsolt et al., 1977). SJW was active in the forced swimming test and produced results similar to those observed with the traditional antidepressants (Barnes et al., 2001; Butterweck et al., 1997).

Many of the models of anxiety are based on the exploratory behavior of animals. The plus-maze test is useful in identifying both anxiolytic and anxiogenic drugs. The model involves the use of a plus-shaped maze constructed to have two open and two enclosed arms. The animal is placed inside the maze, and allowed to explore freely for a short period. The proportion of entries made on to the open arms of the maze and the time spent on the open arms are expressed as a percent of the total time spent on both the open and closed arms of the maze. A treatment that increases the animal's preference for the exposed arms, without changing the total number of arm entries, is believed to be anxiolytic, while a treatment that results in the decreased preference of the animal for the open arms is anxiogenic. A reduction in the total number of arm entries may confound the experiment and may be a reflection of a sedating effect. In this

model, SJW was found to exert effects similar to those of the TCAs and SSRIs (Gambarana et al., 2001).

The potential anxiolytic effect of SJW extract was explored in rats and the extract was found to increase locomotor activity in an open-field test. The extract also demonstrated an anxiolytic effect in a light-dark test (Barnes et al., 2001). Pretreatment of the animal with flumazenil, a benzodiazepine receptor antagonist, was found to block the anxiolytic effects (Barnes et al., 2001).

The administration of SJW to rats prior to unavoidable shock session protected the animals from developing symptoms of unavoidable stress exposure (Gambarana et al., 2001). This effect was comparable to the effects of imipramine, a TCA.

The administration of SJW, fluoxetine or imipramine, to rats exposed to chronic stress reversed stress-induced behavioral deficits (Gambarana et al., 2001). SJW also restored altered dopaminergic transmission in the nucleus accumbens caused by exposure to chronic stress, with results similar to those induced by imipramine and fluoxetine (Gambarana et al., 2001).

A variety of other animal models have been used to evaluate the effects of anxiolytic drugs in rats. These include the elevated T-maze (for inhibitory avoidance and escape behavior), the light/dark transition model and the cat odor test. Flausino et al., (2002) reported that both the acute and chronic administration of SJW produced an anxiolytic effect in these models.

1.7 SJW mechanism of action

The mechanism of action of SJW remains to be conclusively elucidated, although there are a number of potential mechanisms that have been proposed. The acute biochemical effects of SJW extract, including those on receptor binding, microdialysis, enzyme activity, and uptake experiments have been studied *in vitro* and *in vivo*. A number of constituents have been tested for activity in these assays in an attempt to identify the active constituent(s). Long-term effects have also been evaluated, as SJW must be taken for several weeks for the clinical antidepressant effects to become apparent (Jensen et al., 2001).

1.7.1 Hypericin

In 1984, it was reported that hypericin (HYPC) was the constituent responsible for the antidepressant activity of SJW. The

structures of HYPC and pseudohypericin are shown in Figure 2 (page 40). HYPC was shown to inhibit MAO activity *in vitro*. This effect was proposed as the antidepressant mechanism of action, and SJW preparations were standardized with respect to HYPC content (Barnes et al., 2001; Nathan, 2001). It has since been discovered that the concentration required for MAO inhibition *in vivo* is too high to be achieved under physiological conditions (Nathan, 2001; Whiskey et al., 2001) and that SJW is only a weak inhibitor of MAO-A and MAO-B activity (DiCarlo et al., 2001). The antidepressant efficacy observed with a SJW extract devoid of HYPC provides further evidence that this constituent is not likely to be significantly involved in the mechanism of action.

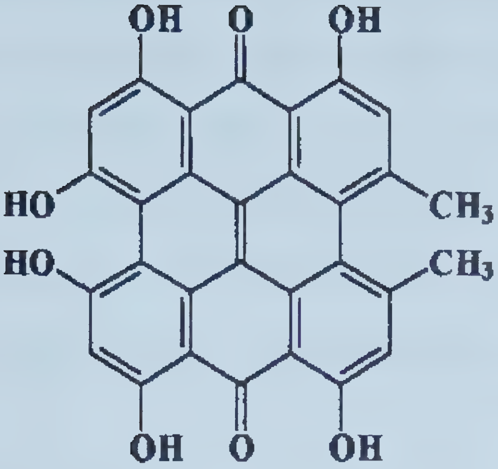
1.7.2 Receptor binding studies

To determine the pharmacological profile of SJW extract, the effects of the total extract and individual constituents were tested at various central nervous system receptors, using radioligand binding techniques. At a concentration of 100μg/mL, SJW extract did not inhibit ligand binding at 5-HT_6 and 5-HT_7, benzodiazepine, sigma, neuropeptide Y (NPY), histamine, neurokinin, or corticotrophin releasing hormone receptors (Simmen et al., 2001), while the extract did interact with DA transporters at higher concentrations. At a

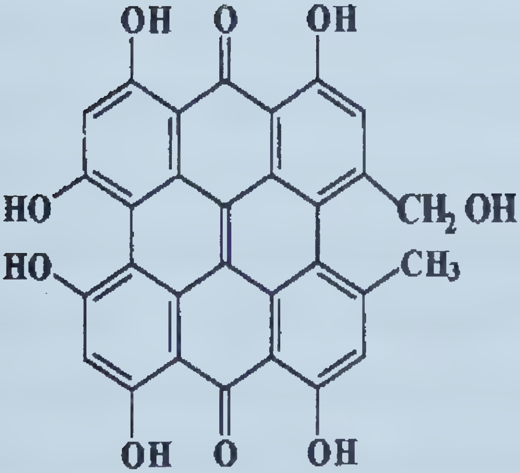
concentration of 1mg/mL, HYPF inhibited ^{3}H-WIN-35428 binding to DA transporters. The IC_{50} value for this effect, however, is higher than that required for the inhibition of synaptosomal reuptake of DA. HYPC had affinities for, sigma, NPY_1 and NPY_2 receptors in the micromolar range; this is of interest since it has been proposed that NPY and sigma receptors may be involved with anxiety disorders, depressive illnesses and alcoholism (Gobbi et al., 2001).

HYPF inhibited binding to opioid and 5-HT receptors with IC_{50} values in the low micromolar range. Biapigenin, a biflavonoid constituent, inhibited binding to the estrogen α-receptor, with an IC_{50} value in the low micromolar range. A crude extract of SJW led to the inhibition of ^{3}H-muscimol binding to the $GABA_A$ receptor complex. It has been proposed that these actions may act in a synergistic manner, and altogether, provide the clinical response (Simmen et al., 2001).

The extracellular levels of 5-HT, NA and DA in the brain have been found to increase following treatment with SJW. These effects are indicative of either stimulation of release and/or inhibition of reuptake of these neurotransmitters. Through the use of microdialysis techniques, *in vivo* extracellular levels of these neurotransmitters



2(a). Hypericin



2(b). Pseudohypericin.

Figure 2. Structures of two naphthodianthrone constituents of SJW. 2(a) is the structure of hypericin, while 2(b) is the structure of pseudohypericin. (Nahrstedt and Butterweck, 1997).

were measured in rat brain regions and monitored in both acute and chronic models of SJW administration. In one *in vivo* microdialysis experiment, the levels of DA and 5-HT were found to increase in the shell region of the nucleus accumbens (Rommelspacher et al., 2001). Reduced levels of DA in the nucleus accumbens have been associated with animal models of depression (Rommelspacher et al., 2001).

1.7.3 SJW and reuptake inhibition

SJW extract was found to inhibit the reuptake of 5-HT, NA, and DA in a dose-dependent manner, in rat brain synaptosomes (Gobbi et al., 2001; Wonnemann et al., 2001). In addition to inhibiting uptake of NA, 5-HT and DA in synaptosomal preparations, SJW has been shown to inhibit uptake of 5-HT in intact astrocytes (Neary and Bu 1999). The extract also demonstrated 5-HT uptake inhibiting activity in neuronal cell cultures from the serotonergic-rich raphé nuclei (Neary et al., 2001). The 5-HT uptake inhibiting effects of the extract were found to be 25 times more potent in neurons compared to astrocytes (Neary et al., 2001). The inhibition of NA uptake by LI 160 was examined in cells derived from a human neuroblastoma cell line enriched with NA transporters (Neary et al., 2001). The extract inhibited the uptake of NA in the cultured cells. HYPF was found to be between 10 and 20 times more potent than the total LI 160 extract at

inhibiting the uptake of neurotransmitters in astrocytes and neuronal cells. This finding is consistent with the chemical composition of SJW extract, as HYPF is found in the range of 4 to 5% (Neary et al., 2001).

The clinical benefits of SJW are delayed and it may take several weeks before the symptoms of depression begin to subside. This phenomenon is also characteristic of the traditional antidepressants. The effects of continuous, repeated treatment with SJW have been examined and it was discovered that SJW extract stimulates a sustained activation of extracellular signal regulated protein kinase (Erk). Erk is a key component of a complex signal transduction pathway that may be associated with gene expression. This discovery has led to speculation that SJW may affect not only rapidly-acting biochemical mechanisms (such as reuptake inhibition) but also slower-developing, more long-term mechanisms (such as gene expression) related to depressive disorders (Neary et al., 2001).

1.7.4 Hyperforin

HYPF has recently been proposed as the constituent most likely responsible for the antidepressant activity of SJW (Muller et al., 2001; Wonneman et al., 2001; Wurglics et al., 2001) and has demonstrated

activity in a number of biochemical and behavioral models of antidepressant activity (Chatterjee et al., 1998).

HYPF has the chemical formula $C_{35}H_{52}0_4$ (Orth et al., 1999) and is one of the main constituents of SJW, accounting for between 2 and 4% of the dried herb. The structure of HYPF is shown in Figure 3. In rats and humans the administration of HYPF induced EEG changes that resemble changes induced by the SSRIs (Dimpfel et al., 1998). Changes in the alpha frequency occurred, and such changes are believed to be an indicator of serotonergic and dopaminergic modulation (Dimpfel et al., 1998).

HYPF has been shown to have moderate affinity for the sigma receptor, but concentrations required for this effect are in the micromolar to millimolar range (Neilson, 1998). The SSRIs and MAOIs have recently been shown to exhibit moderate affinity for the sigma receptor, and chronic treatment with these antidepressants led to a down-regulation of such receptors (Neilson, 1998).

HYPF exhibits potent inhibition of 5-HT, NA and DA uptake, and extracellular concentrations of these neurotransmitters in rat brain regions were elevated following intraperitoneal injection of HYPF

(Wonneman et al., 2001). HYPF potently inhibited the uptake of 5-HT, NA and DA in a synaptosomal preparation of rat striatum, with IC_{50} values in the nanomolar to low micromolar range (DiCarlo et al., 2001; Jensen et al., 2001).

HYPF also inhibits GABA and GLU uptake in rat brain synaptosomes. The uptake inhibition of GLU requires concentrations slightly higher than those required for GABA uptake inhibition (Wonnemann et al., 2001). The inhibition is non-competitive in nature, and simple substrate competition for ligand binding sites has been ruled out as the mechanism of action (Wonneman et al., 2001). The uptake inhibition was not caused by non-specific damage, as uptake was partially restored following a wash-out of HYPF (Wonneman et al., 2001). The mechanism is not likely associated with specific binding to transporters, but instead due to a general effect on transport function (Wonneman et al., 2001).

HYPF inhibited binding of ^{3}H-WIN-35428 to DA transporters with an IC_{50} of 2.6ug/mL (Gobbi et al., 2001). This concentration is higher than the concentration required for uptake inhibition of DA, NA and 5-HT. A similar effect was shown with the 5-HT transporter, indicating

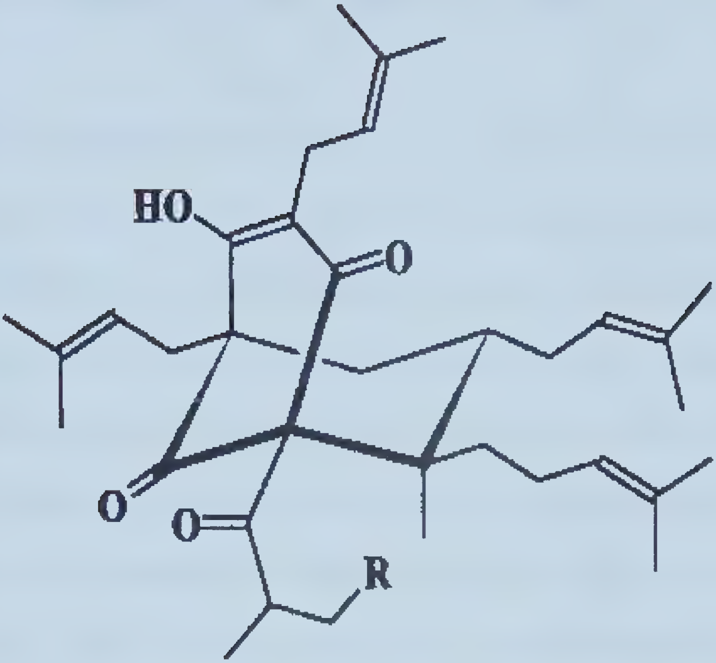


Figure 3. Structure of hyperforin and adhyperforin. The basic structure is shared between these two phloroglucinols, while the side chain varies. R=H for hyperforin; R=CH_3 for adhyperforin (Nahrstedt and Butterweck, 1997).

that the uptake inhibition is not due to a direct effect on the transporter (Gobbi et al., 2001; Gobbi et al., 1999).

Further evidence that HYPF is the constituent involved in the mechanism of action of SJW comes from conventional pharmacological models indicative of antidepressant activity that showed a strong correlation between potency of SJW effects and HYPF content (DiCarlo et al., 2001). The efficacy of SJW in animal models of depression, such as the forced swimming test, has been linked to HYPF content, with higher HYPF-containing extracts demonstrating greater efficacy. In the acute escape deficit model, HYPF demonstrated a potency of approximately ten times that of the total SJW extract in protecting animals from the effects of unavoidable stress (Gambarana et al., 2001).

The broad-spectrum effect of HYPF on neurotransmitter reuptake may be mediated by an elevation of intracellular Na^+ concentration. It is believed that this occurs via activation of Na^+ conductive pathways not yet fully identified (Muller et al., 2001), but most likely to be ion channels. HYPF is described as the first member of a new class of

compounds with a preclinical antidepressant profile due to a completely novel mechanism of action (Muller et al., 2001).

1.7.5 Other mechanisms

Although there is strong evidence implicating a role for HYPF in the antidepressant activity of SJW, it is likely not the lone active constituent. In an animal model of antidepressant activity, the administration of an extract of SJW devoid of HYPF was compared to an extract containing 3.8% HYPF (Wonnemann et al., 2001). Although the activity was higher for the HYPF-containing extract, the HYPF-free extract did exhibit antidepressant activity. It has been proposed that these effects are caused by an unidentified constituent, or that the constituents may act in a synergistic manner that has not yet been revealed (DiCarlo et al., 2001; Wonnemann et al., 2001). Adhyperforin, a derivative of HYPF, exhibits reuptake potency similar to that of HYPF, although it is found in much smaller quantities in the extract (Wonnemann et al., 2001). The oligomeric procyanidin fraction also demonstrated weak to moderate reuptake inhibition although it is believed that the *in vivo* concentrations required for such activity are likely not attainable physiologically (Wonnemann et al., 2001). The antidepressant mechanism of action of SJW may also be related to effects on sigma receptors. In the forced swimming test,

the anti-immobility effects of an extract of SJW containing 0.3% HYPC and 3.8% HYPF were reversed by intraperitoneal treatment with 20mg/kg rimcazole, a sigma receptor antagonist (Pancoka et al., 2000).

1.7.6 Long-term changes

The delayed onset of therapeutic activity may be related to long-term, adaptive effects on neuronal function, and such effects may involve changes in gene expression (Neary et al., 2001). Chronic treatment with SJW has been shown to produce adaptive changes in β-adrenoceptor density and 5-HT$_1$ and 5-HT$_2$ receptor densities in rat frontal cortex (DiCarlo et al., 2001). A downregulation of β$_1$-adrenoceptors, which is frequently used as a biological marker of antidepressant activity (Muller et al., 1997), occurs after chronic administration of SJW. However, chronic treatment with SJW led to an up-regulation of postsynaptic 5-HT$_{1A}$ and 5-HT$_2$ receptors, which in the latter case contrasts with the downregulation observed with the TCAs and MAOIs (Muller et al., 1997) (see Table 2).

The effects of both acute and long-term administration of SJW and HYPC on regional monoamine levels were examined in rat brain. The levels of 5-HT were found to increase in the hypothalamus,

Table 2. The long-term effects of antidepressant treatment, electroconvulsive therapy and SJW administration.

TYPE OF RECEPTOR	ANTIDEPRESSANTS	ECT	HYPERICUM AND CONSTITUENTS
α_2-adrenoceptors	Decreased		
α_1-adrenoceptors	Increased		
β-adrenoceptors	Decreased	Decreased	Decreased (extract and HYPF
5-HT_2 receptors (post-synaptic)	Decreased	Increased	Increased (extract and xanthones) Decreased (HYPF)
5-HT_{1A} (post-synaptic)	Increased		Increased (extract and HYPF)
5-HT_{1A} (autoreceptors)	Decreased (for SSRIs and MAOIs)	increased	
Dopamine D_2 receptors	Decreased		Decreased (xanthones)
$GABA_A$ receptors	Increased		Increased (xanthones)

(Adapted from Nathan, 2001)

following treatment with either SJW, HYPC or imipramine, a TCA (Butterweck et al., 2002). Following 8 weeks of treatment,with either imipramine, SJW or HYPC, the rate of 5-HT turnover was decreased in both the hypothalamus and the hippocampus.

It has been proposed that alcoholism and depression share some neurochemical characteristics, such as low brain levels of 5-HT. Rezvani et al. (1999) evaluated voluntary alcohol intake using two animal models of human alcoholism. The animals received either a single oral dose of vehicle or SJW. The administration of SJW was shown to significantly reduce the alcohol intake by the animals, and this effect showed little tolerance following 15 consecutive days of treatment. These researchers therefore recommended that SJW be evaluated clinically as a potential treatment for alcoholism.

1.8 Analytical procedures

1.8.1 General spectrophotometric principles

Spectrophotometry is used to measure the amount of light that an analyte absorbs. A beam of light, at a specified wavelength, is passed through the sample containing the analyte and the intensity of the light reaching a detector is measured. The beam of light is composed of a stream of photons and when these photons encounter a molecule in the analyte the photon may be absorbed. As photons in the beam of light are absorbed the intensity of light reaching the detector is reduced. The extent to which an analyte absorbs light depends on the wavelength of light. Monochromatic light is used, in which all of the photons have the same wavelength. An absorbance spectrum is a plot of absorbance versus wavelength and is plotted using the wavelength at which the absorbance is greatest. The wavelength is characteristic of each compound and can provide information on the electronic structure of the analyte.

The cell path length is the length of sample that the light must pass through. The concentration of analyte in the sample will affect absorbance and at higher concentrations more photons are absorbed. Thus, absorbance will vary linearly with concentration and path length.

Spectrophotometry is useful in the measure of enzymatic reactions. The reaction can be monitored as the absorbance will change in relation to substrate concentration. The reaction can be observed over time, and a continuous plot of absorbance can be constructed as substrate concentration changes via enzymatic activity.

In the enzyme assay described in this thesis, the effect of SJW on GABAse, a bacterial form of GABA-T, was investigated using spectrophotometry. The reaction described in Figure 1 (page 11) was monitored. As GABA is metabolized by GABA-T, SSA is produced. SSA is then metabolized by SSADH to SSA, with the conversion of $NADP^+$ to NADPH occurring simultaneously. The production of NADPH is monitored, and the resultant change in optical density is measured.

1.8.2 Principles of high performance liquid chromatography

The basic principle by which chemical separation is achieved is common to all types of chromatography. A stationary phase, consisting of particles of a substance such as resin, cellulose, or alumina, packed into a tube through which the mobile phase will flow. In high performance liquid chromatography (HPLC), the mobile phase is a liquid and it is pumped through an attached column at high

pressure. There are two flow gradients that can be utilized. Isocratic flow maintains a constant mobile phase composition, while gradient flow allows for the mobile phase composition to be changed in either a continuous, or stepwise, manner (Holman et al., 1993). The mobile phase is used to carry the sample, containing the analyte(s), into the column. Once inside the column, the compounds are separated from each other due to a differential equilibrium between the mobile phase, the column and the analyte itself (Feldman et al., 1997). The effluent from the column is then analyzed using a detector system. A particular analyte can be identified as each compound has a characteristic retention time.

In liquid chromatography, systems can be either normal or reverse phase systems. A normal phase system utilizes a stationary phase that is more polar than the mobile phase. A reverse phase system uses a separation system in which the mobile phase is more polar than the stationary phase. The reverse phase system will elute polar analytes first and is often favored as many biological compounds are polar. A reverse-phase system was used in the HPLC experiments described in this thesis.

The stationary phase is composed of an organic substance covalently bound to the surface of a support particle. In most cases, silica is the support particle of choice (Burtis et al., 1987). The organic moiety of the stationary phase may be either polar or non-polar. Non polar organic moieties include octadecylsilane (ODS or C-18) and polar groups include cyanopropyl (CN), aminopropyl (NH2) and glycidoxypropyl (diol) silanes (Burtis et al., 1987).

In the HPLC analysis of the glutamate formed in the GABAse assay, described in this thesis, a fluorescence detector was utilized to detect GLU and GABA as they eluted from the column. Fluorescence detection is used to detect compounds that are either naturally fluorescent or compounds that have been converted to fluorescent derivatives (Baker et al., 1985). The samples were derivatized with *o*-phthalaldehyde (OPA) prior to injection onto the column. In fluorescence detection, ultraviolet radiation is produced by a light source of a particular wavelength (excitation wavelength) and directed onto the sample being analyzed. As the light passes through the sample, light is emitted at a specific wavelength (the emission wavelength) and reaches the detector (Baker et al., 1985). The number of photons emitted from the sample will be proportional to the concentration of fluorescent substance that is in the sample.

1.9 Objectives of the research described in this thesis

As mentioned previously, there has been increased interested in recent years in the possible role in depression of GABA, an amino acid known to play a role in the action of anxiolytic drugs. Since SJW has been reported to have antidepressant and anxiolytic efficacy, the purpose of the research described in this thesis was to study the effects of SJW and several of its constituents on two factors which influence GABA markedly, namely its catabolism and its uptake. Therefore, the main objectives were:

1. To investigate the effects of SJW on GABA-T activity *in vitro*.

   This was done by studying inhibition of GABAse (a bacterial form of the enzyme containing GABA-T and SSADH) and by utilizing an HPLC assay to study GABA-T indirectly by measuring GLU levels.

2. To investigate the following aspects of the effects of SJW on GABA uptake *in vitro*:
   a. the effect of CVT (CV Technologies) SJW.
   b. the effect of SJW constituents.
   c. the effect of HYPF.
   d. the effect of several commercially available SJW preparations.

2. MATERIALS

2.1 Instrumentation and Apparatus

A Powerwave$_x$ spectrophotometric plate reader (Bio-Tek Instruments Inc., Winooski, VT) was used to analyze the activity of GABAse. UV transmitting plates were utilized. Data were processed by a Pegasus Pentium 2 computer, equipped with KC4 software (Bio-Tek Instruments).

The HPLC utilized in the analysis of GABA and GLU levels was the Waters 2690 Separations Module (Waters Associates, Milford, MA), equipped with a Waters 474 Fluorescence Scanning detector (Waters Associates, Milford, MA). A Spherisorb ODS2 column C18 (3.9 x 150mm I.D., 5 micron particle size) was used in this experiment. Data were processed by a Pegasus Pentium 2 computer, 400MHz (Pegasus Computer Company, Edmonton, AB), using Waters Millenium software.

A Beckman scintillation counter was utilized to measure radioactivity of samples. Data were processed using a Pegasus Pentium 2 computer.

2.2 Equipment

A Mettler AE 160 electronic balance (Mettler Instrument Corporation, Highstown, NJ) was used to weigh samples, tissues and chemicals.

A Brandel Cell Harvester was utilized in the GABA uptake assay for filtration of samples.

A Sorvall GLC-2B General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE) was used to prepare samples requiring centrifugation at low speeds.

Microfuge tubes were centrifuged in a Beckman Model J-21B Centrifuge (Beckman Instruments, Palo Alto, CA), up to a speed of 10,000 rpm.

The mobile phases for the HPLC experiments were filtered and degassed, using a Millipore Filtering system (Millipore Corporation, Bedford, MA). Mobile phase was forced through a Nylon Filter Membrane (0.2uM pore size, 47mm diameter) (Phenomenex, Torrance, CA), under vacuum.

Dirty glassware was rinsed thoroughly with tap water and subsequently placed in the dishwasher (Miele Electronics 6715). Sparkleen detergent (Fisher Scientific) was added to the dishwasher. The test tubes were first sonicated (ultra-sonic cleaner, Mettler Electronics) in a 2 – 5% solution of Decon 75 (BDH chemicals). The tubes were then rinsed and placed into the dishwasher, where they were rinsed with double-distilled water. All glassware was subsequently air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, USA.

A Fisher Isotemp waterbath (Fisher Scientific, Nepean, ON) was used in experiments requiring incubation at 37°C.

The pH of solutions was measured with an Accumet pH Meter 915, with an Accumet probe (Fisher Scientific, Nepean, ON).

A McIlwain tissue chopper was used to prepare the cortical prisms for the uptake experiments.

A Thermolyne Maxi Mix (Sybron/Thermolyne Instruments, Dubuque, IO) was used to mix samples and solutions.

Double-distilled water was produced using a Mega-Pure Three Liter Automatic Water Still (Corning Waterware, Corning, NY). The double-distilled water was further purified by passing through a Mixed Bed Organic Removal cartridge (Fisher Scientific, Palo Alto, CA).

2.3 Chemicals

CHEMICAL	SUPPLIER
Acetonitrile	Fisher Scientific (Nepean, ON)
γ-Aminobutyric acid	Sigma (St. Louis, MO)
Aminooxyacetic acid	Sigma
Ascorbic acid	Fisher Scientific
Calcium chloride	Fisher Scientific
CVT SJW	CV Technologies
Ethylenediaminetetraacetate, disodium salt	Fisher Scientific
GABAse	Sigma
Glucose	Sigma
Glutathione	Sigma
Glutamate	Sigma
^{3}H-GABA	NEN Products (Boston, MA)
Hydrochloric acid	Fisher Scientific
Hyperforin	CV Technologies
Hypericin	Sigma
Isoquercitrin	Indofine Chemicals
Kaempferol	Sigma
α-Ketoglutarate	Sigma
Magnesium sulfate	Fisher Scientific

Methanol	Fisher Scientific
β-Nicotinamide adenine dinucleotide phosphate	Sigma
Nipecotic acid	BDH Chemicals
Phenelzine	Sigma
o-Phosphoric acid	Fisher Scientific
o-Phthalaldehyde	Pierce Chemicals (Rockford, IL)
Potassium chloride	Fisher Scientific
Pseudohypericin	Indofine
Quercetin	Sigma
Quercitrin	Sigma
Ready Safe scintillation fluid	Beckman Dickinson
Sodium chloride	Fisher Scientific
Sodium hydroxide	Fisher Scientific
Sodium pyrophosphate, anhydrous	Fisher Scientific
Tetrahydrofuran	Fisher Scientific
TRIS	Fisher Scientific

3. METHODS

3.1 Analysis of the effects of PLZ and SJW on GABAse activity

3.1.1 Preparation of Solutions and Samples

The incubation buffer used was a 100mM NaH_2PO_4 solution, prepared by adding 2.659g anhydrous tetrasodium pyrophosphate to 100mL double distilled water. To assist in dissolving the powder, the preparation was stirred and heated slightly, by a heat/stir plate. Once cooled, the buffer was brought to a pH of 8.4, with *o*-phosphoric acid, and kept refrigerated in a sealed bottle until required for use.

The GABAse buffer was prepared fresh daily by adding 1.5mg glutathione (GSH) to 10ml of sodium pyrophosphate buffer, for a 500μM concentration of GSH. This buffer was kept cold until required for use.

The enzyme was prepared in a 1.5mL microfuge tube by adding 1.25mL GABAse buffer to each unit of enzyme, to reach an enzyme concentration of 0.8U/mL. This preparation was mixed gently by inversion of the tube, and subsequently placed on ice, and kept cold and protected from light, until required for use. The enzyme was prepared fresh daily.

α-Ketoglutarate was prepared by dissolving 1.7mg in 2.5mL double distilled water. GABA was prepared, at a concentration of 20mM, by dissolving 5.2mg in 2.5mL double distilled water. NADP was prepared daily, by dissolving 19.1mg in 2.5mL double distilled water.

On the day of the assay, the reaction buffer was prepared by adding 2.5mL of GABA solution and 2.5mL of NADP solution to 10mL of incubation buffer, and subsequently placed on ice until required for use.

A crude extract of SJW, provided by CV Technologies (Edmonton, Alberta) was utilized throughout the experiments described. The raw plant material was extracted with methanol and subsequently dried via evaporation. The powdered extract was kept refrigerated and protected from light. On the day of the assay, a stock solution of CVT SJW was prepared by dissolving 3mg of extract in 1mL of incubation buffer. This mixture was vortexed and diluted to produce a range of SJW concentrations. All SJW samples were centrifuged at 1000g for 2 minutes, to remove particulate matter. The samples were kept on ice, protected from light, until required for use in the assay.

A stock solution of 1 x 10^{-3}M PLZ was prepared by dissolving PLZ in double distilled water. This stock solution was diluted, in incubation buffer, to produce a range of concentrations. The samples were kept on ice, protected from light, until required for use in the assay.

3.1.2 Procedure for the analysis of GABAse activity

The assay was performed in 96-well UV-transmitting plates. Each sample was tested in quadruplicate. All wells received 25μl of GABAse. The blanks and controls received 50μl of sodium pyrophosphate buffer, while the remaining wells received 50μl of the test drug. Reaction buffer (150μl) was then added to all wells. As rapidly as possible, 25μl α-ketoglutarate was added to all wells, with the exception of the blanks, which received 25μl sodium pyrophosphate buffer. The plate was immediately placed into the platereader, at a temperature of 37°C, and change in optical density was measured over a period of 40 minutes. Optical density changed as $NADP^+$ was converted to NADPH.

3.1.3 Instrumentation

All samples, in UV-transmitting plates, were analyzed by a platereader. The wavelength was set at 340nm and the platereader was set to record optical density every 30 seconds, over a 40-minute

duration. Data were recorded by a Pegasus Pentium computer, using the KC4 software program.

3.2 Analysis of the effect of SJW constituents on GABAse activity

3.2.1 Preparation of solutions and samples

The solutions used in this assay were prepared as described in section 3.1.1 above. All solutions were kept on ice, protected from light, until required for use in the assay.

A variety of SJW constituents were tested for inhibitory effects on GABAse activity. Hypericin, pseudohypericin, hyperforin, quercetin, quercitrin and kaempferol were prepared in incubation buffer, at a concentration of 1 mg/mL, to attain a final assay concentration of 200μg/mL. All samples were vortexed, centrifuged at 1000g for 2 minutes, and kept on ice, protected from light, until required for use in the assay.

3.3 Analysis of the effects of PLZ and SJW on GLU production in the GABAse assay

3.3.1 Solution Preparation

The solutions required for this assay were prepared as described in section 3.1.1, with some minor modifications. The incubation buffer, GABAse buffer, GABAse preparation, NADP, GABA, α-ketoglutarate and the reaction mixture were all prepared as described. The remaining solutions were prepared as described below.

Mobile Phase A was prepared by dissolving 8.755g NaH_2PO_4 in 900mL double distilled water. Once dissolved, 240mL methanol, 20mL acetonitrile, and 10mL tetrahydrofuran (THF) were added. The pH was adjusted to 6.2 with 10N sodium hydroxide (NaOH).

Mobile Phase B was prepared by dissolving 6.566g NaH_2PO_4 in 1340mL double distilled water. Once dissolved, 1110mL methanol and 60mL THF were added. The pH was adjusted to 6.2 with 10N NaOH. Both mobile phases were filtered and degassed, under vacuum, using 0.2μm filters.

Stock solutions of the amino acids GABA and GLU were prepared as 1mg/mL concentrations and stored on ice, protected from light.

CVT SJW extract was prepared as a stock solution in incubation buffer, at a concentration of 5mg/mL. This solution was diluted to attain final assay concentrations of 1.5mg/mL, 0.5mg/mL, and 0.15mg/mL. All samples were briefly mixed by vortex and subsequently centrifuged for 2 minutes at 1000g. These samples were stored on ice, protected from light.

PLZ was prepared by diluting the 1 x 10^{-3}M stock solution to attain final assay concentrations equal to 1x 10^{-4}M, 5 x 10^{-5}M, and 1 x 10^{-5}M. The PLZ samples were kept on ice, protected from light, until required for use.

A stock solution of aminooxyacetic acid (AOAA) was prepared, in double distilled water, a concentration of 1 x 10^{-3}M. This solution was further diluted in incubation buffer to a concentration of 5 x 10^{-5}M. The stock solution was kept on ice and protected from light, until required for use.

3.3.2 Procedure for analysis of GLU levels in the GABAse assay

The HPLC analysis involved a previously published method (Parent et al., 2001), with some modifications. The experiment was

performed in 1.5mL plastic microfuge tubes on ice. All tubes received 25μl of GABAse preparation. The blank and control tubes then received 50μl of incubation buffer, while the remaining tubes received 50μl of the test drug. All tubes received 150μl of the reaction mixture (containing NADP and GABA, in buffer). The reaction was initiated by adding 25μl α-ketoglutarate to all tubes, with the exception of the blanks, which received 25μl incubation buffer. The tubes were placed into a 37°C water bath, for an incubation period of 40 minutes. Upon completion of the incubation, the tubes were removed from the water bath and an aliquot of 100μl was removed from each and placed into HPLC vials.

3.3.3 Instrumentation

All samples were analyzed using the Waters Alliance 2690XE Separations Module system, equipped with a Waters 474 Fluorescence detector, set at gain 10. The excitation wavelength was 260nm, while the emission wavelength was 455nm. The guard column utilized was a Waters μBondpak C18 column. A Waters Novapak ODS2, C18 (3.9 x 150mm I.D., 5 micron particle size) was used as the column. OPA was replaced daily, from a refrigerated stock supply stored under nitrogen. A 5μl aliquot was removed from each sample and 5μl of OPA were added to the sample, while it was held in the loop for 1.5 minutes prior

to injection onto the column. As derivatized amino acids are unstable over time, OPA is added to the sample so that derivitization occurs immediately prior to injection (Parent et al., 2001). The mobile phase was composed of a gradient set initially at 50% phase A and 50% phase B. The flow rate was set at 0.5mL per minute. The gradient was changed to 100% phase B after 10 minutes. At 20 minutes, the flow rate was returned to the initial conditions and maintained for at least 20 minutes, to condition the column. The total run time was 40 minutes.

3.4 Analysis of ^{3}H-GABA uptake by SJW

3.4.1 Preparation of solutions and samples

The incubation medium (IM) was prepared in a 4 L plastic bottle, rinsed with both hot water and double distilled water. An approximate volume of 3900mL of double distilled water was added to the bottle. The following chemicals were added, with final concentrations shown in parentheses: 28.757g NaCl (123mM), 1.490g KCl (5mM), 1.203g $CaCl_2$ (2.7mM), 0.578g $MgSO_4$ (1.2mM), 9.696g TRIS (20mM), 0.704g ascorbic acid (1mM) and 7.2g glucose (10mM). The final volume was adjusted to 4 L with double distilled water. The pH was adjusted to 7.5, using 5M HCl, prior to the addition of glucose. AOAA, 0.1mM, was added to the IM to inhibit endogenous GABA-T.

All samples were prepared in IM. Nipecotic acid, a known inhibitor of GABA uptake, was prepared as a stock solution of 100 mM and was subsequently diluted to attain a final assay concentration of 10μM.

CVT SJW extract was prepared as a stock solution of 10mg/mL. This sample was vortexed to mix, and subsequently diluted to produce concentrations of 1mg/mL, 0.3mg/ml, 0.1mg/mL, and 0.03mg/mL. All samples were centrifuged at 1000g for two minutes, stored on ice, and protected from light until required for use.

3.4.2 Preparation of the tissue suspension

On the day of the assay, a naïve rat was sacrificed by decapitation and the brain was rapidly removed. Cortical tissue was dissected out and placed into ice-cold IM. The tissue was weighed and 1.25mL of IM were added to each mg of tissue. The tissue was chopped into fine prisms, using a McIlwain tissue chopper. As the tissue was chopped, it was kept cold and moist by bathing in ice-cold IM. The tissue was chopped through once, rotated by 90° and chopped through once more, producing tissue prisms of 0.1mm x 0.1mm x approximately 2mm. A Pasteur pipette was used to suspend the tissue prisms in the ice-cold IM. The tissue suspension,

submerged in an ice bath, was placed on a stir-plate and the homogeneity of the suspension was maintained by gentle stirring. The tissue suspension remained viable for up to 4 hours.

3.4.3 Procedure for analysis of ^{3}H-GABA uptake

The assay for ^{3}H-GABA uptake was performed in borosilicate test tubes submerged initially in an ice bath. The assay was performed in quadruplicate. The blank tubes received 800µl of IM, while all the remaining tubes received 800µl of tissue suspension. The blank and control tubes then received 100µl of IM, while 100µl of the test sample was added to each of the remaining tubes. All tubes were placed into a 37°C water bath, for a 5-minute incubation period. During the incubation period, the filtration apparatus was prepared by rinsing with distilled water and warm IM. Following the incubation period, the tubes were returned to the ice-bath and 100µl of ^{3}H-GABA was added to all tubes. The tubes were then replaced into the warm water bath, and incubated for 10 minutes. Immediately following this incubation period, the tubes were rapidly filtered, using the Brandel Cell Harvester. The filters were removed and placed into vials containing 4mL Ready Safe scintillation fluid. The vials remained at room temperature for 24 hours prior to counting radioactivity with a

Beckman Scintillation Counter. The Cell Harvester was thoroughly rinsed with distilled water, for at least five to seven minutes.

3.4.4 Instrumentation

All samples were analyzed for radioactivity using a Beckman Scintillation counter, equipped with a program that counts tritium. The data were recorded by computer, using Radiocount software.

3.5 Analysis of the effects of SJW constituents on ^{3}H-GABA uptake

The solutions required for this assay were prepared as described in section 3.4.1. The procedure described in section 3.4.2 was followed.

A variety of SJW constituents were tested in the ^{3}H-GABA uptake assay. Hypericin, pseudohypericin, hyperforin, quercetin, quercitrin, isoquercitrin and kaempferol were prepared as 3mg/mL stock solutions and kept on ice, protected from light, until required for use.

3.6 Analysis of the effects of hyperforin on ^{3}H-GABA uptake

The solutions required for this assay were prepared as described in section 3.4.1. The procedure followed is outlined in section 3.4.2.

A range of concentrations of HYPF was prepared. The concentrations chosen correspond to the average percentage of hyperforin found in the total SJW extract.

3.7 Analysis of the effects of various SJW brands on ^{3}H-GABA uptake

The solutions required for this assay were prepared as described in section 3.4.1. The procedure followed the details in section 3.4.2.

The commercial SJW extracts were prepared as capsules or tablets. One entire dose, either one capsule or one tablet, was used to prepare a stock solution for each brand. The tablets were crushed into a fine powder, while the capsules were pulled apart to release the powdered SJW extract. The powder was then dissolved in IM and vortexed. The stock mixture was diluted to attain a final concentration equal to 300μg/mL. All samples were centrifuged at 1000g for 2 minutes, and subsequently placed on ice, protected from light, until required for use.

3.8 Statistical Analysis

The effects of the various treatments on GABAse activity and on ^{3}H-GABA uptake were evaluated as percentages of control activity and

uptake. The results are graphically represented. ANOVA was performed within each set of experiments. Post-hoc analysis was performed when $p<0.05$, using a Neuman-Keuls test.

4. RESULTS

4.1 Effects of a known GABA-T inhibitor and SJW on GABAse activity

The effect of PLZ on GABAse activity was observed as a decrease in optical density change over time. A decrease in optical density change was also observed with SJW. This change provided an indirect measure of inhibition of enzyme activity and the inhibition was dose-related with both PLZ and SJW. The blank wells showed no change in optical density. The results are displayed graphically in Figure 4.

4.2 Effects of SJW constituents on GABAse activity

The various constituents of SJW tested, including hypericin, pseudohypericin, hyperforin, quercetin, and kaempferol, appeared to have no inhibitory effect on GABAse activity at the concentrations tested. The effect of each constituent on GABAse activity is displayed in Figure 5.

4.3 Effects of PLZ, SJW and AOAA on GLU levels, measured by HPLC

A typical chromatogram of a control in the GABAse assay is shown in Figure 6. In the presence of PLZ, a known inhibitor of GABA-T, the amount of GLU was lower than that of the control. AOAA, a potent

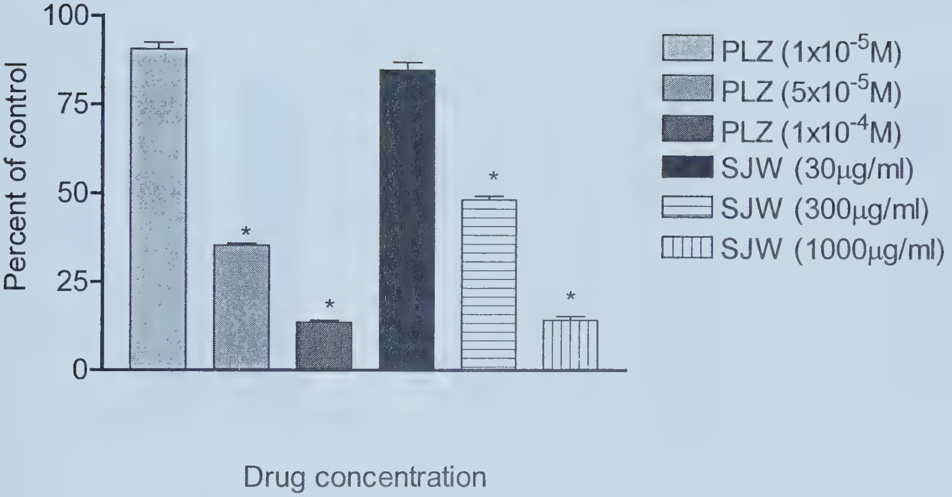


Figure 4. The effect of phenelzine (PLZ) and St. John's wort (SJW) on GABAse activity *in vitro*. Results are expressed as means +/-SEM (N=4) *$P<0.001$.

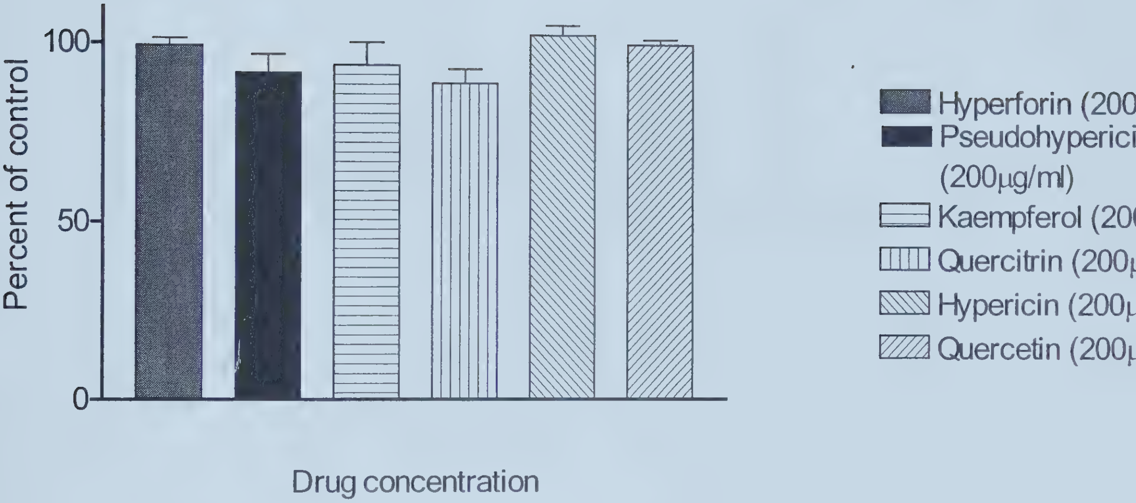


Figure 5. The effect of various constituents of SJW on GABAse activity *in vitro*. Results are expressed as means +/-SEM (N=4).

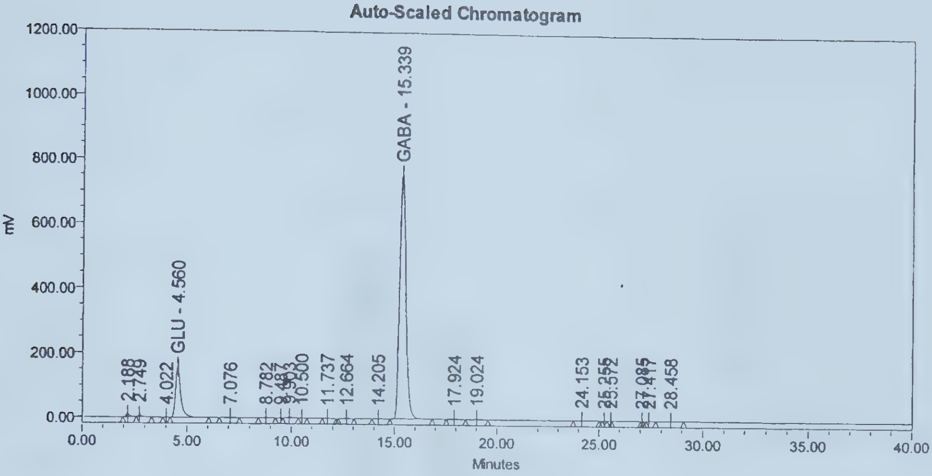


Figure 6. Representative HPLC chromatogram for the CONTROL sample in the GABAse assay.

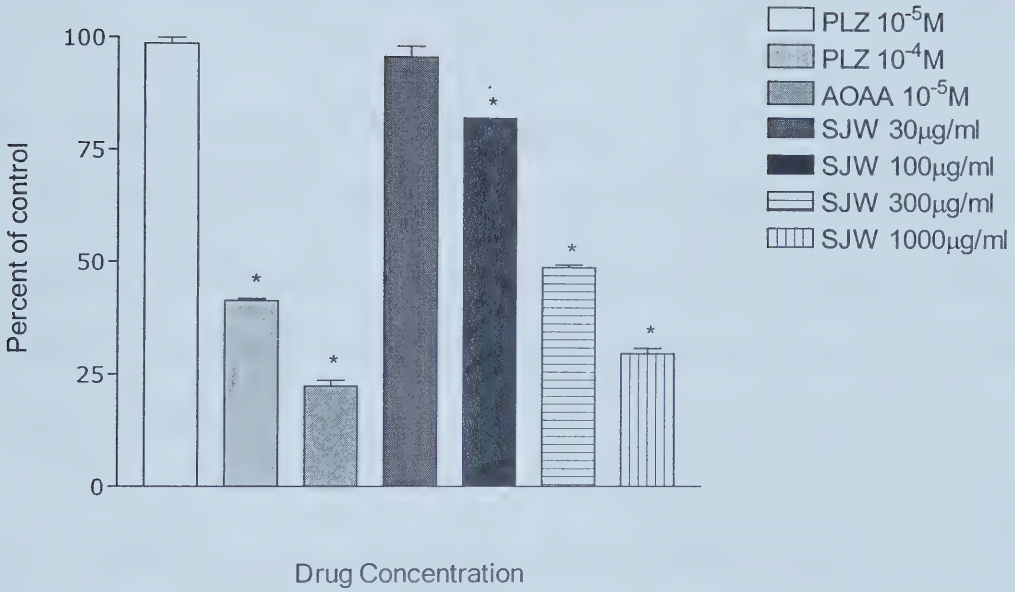


Figure 7. The effect of PLZ, AOAA, and SJW on the production of glutamate in the GABAse assay. Results are expressed as means +/-SEM (N=4).*P<0.001.

GABA-T inhibitor, also inhibited the production of GLU. The amount of GLU produced in the presence of SJW was lower than the GLU production in the control sample, and this effect of SJW was dose-related. The results are graphically represented in Figure 7.

4.4 Effects of SJW on ^{3}H-GABA uptake

The effect of SJW on the uptake of ^{3}H-GABA is represented graphically in Figure 8. All concentrations of SJW tested caused significant inhibition of GABA uptake as did nipecotic acid, a known inhibitor of GABA uptake.

4.5 Effects of SJW constituents on ^{3}H-GABA uptake

The effects of various SJW constituents on the uptake of ^{3}H-GABA are represented graphically in Figure 9. At a concentration of 1000g/mL there was no significant inhibition caused by rutin, HYPC, quercetin, quercitrin, isoquercitrin, pseudohypericin or kaempferol. However, at a much lower concentration, HYPF caused significant inhibition of uptake (see Figure 10).

4.6 Effects of HYPF on ^{3}H-GABA uptake

The effects of a range of HYPF concentrations on ^{3}H-GABA uptake are shown in figure 10. HYPF caused significant inhibition at all concentrations tested and was more potent as an inhibitor than nipecotic acid.

4.7 Effects of Various SJW brands on ^{3}H-GABA uptake

The effects of a variety of different commercially available SJW preparations are shown in Figure 11. Three of the brands (CV Technologies, Webber, and Life Brand) caused significant and similar inhibition of GABA uptake. The Jamieson and Sundown brands did not cause any significant inhibition.

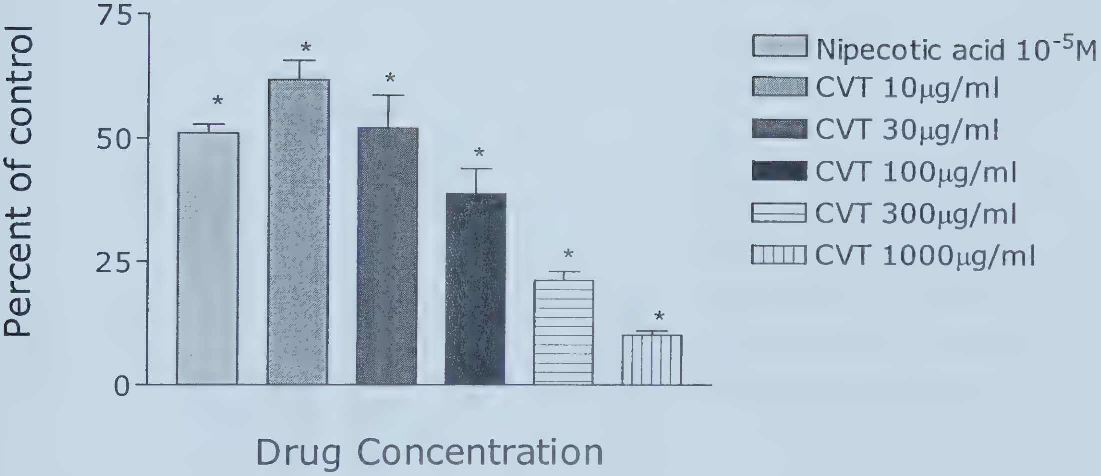


Figure 8. The effect of nipecotic acid and CVT SJW on

^{3}H-GABA uptake. Results are expressed as +/-SEM (N=4).*$p<0.001$

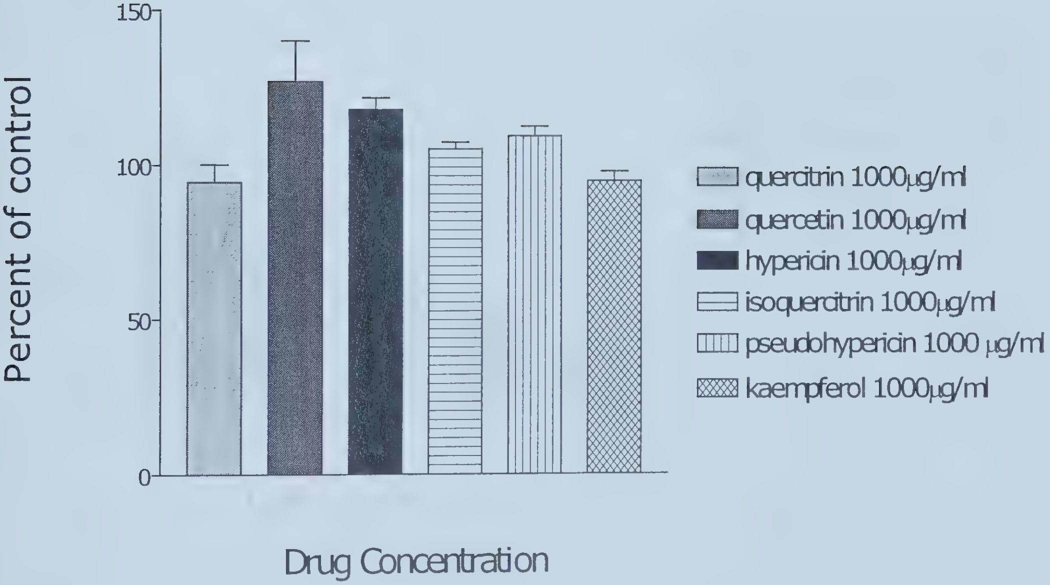


Figure 9. The effect of various constituents of SJW on the uptake of ^{3}H-GABA by rat cortical prisms. Results are expressed as means +/-SEM (N=4).*P<0.001. The results for hyperforin are shown in Figure 10.

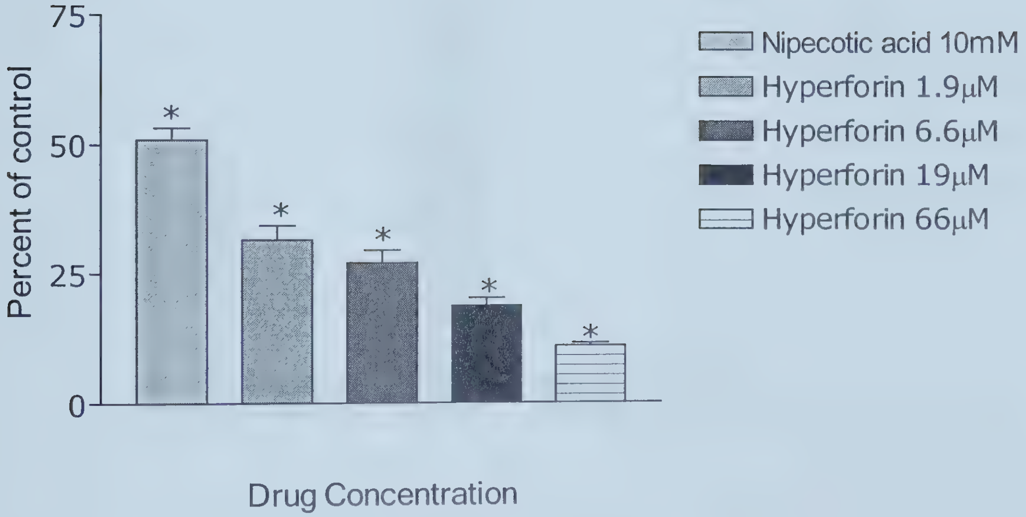


Figure 10. The effect of hyperforin on the uptake of ^{3}H-GABA uptake by rat cortical prisms. The concentrations shown for hyperforin (1.9 – 66μM) correspond to 1.0 – 35.4 μg/ml. Results are expressed as means +/-SEM (N=4).*P<0.001.

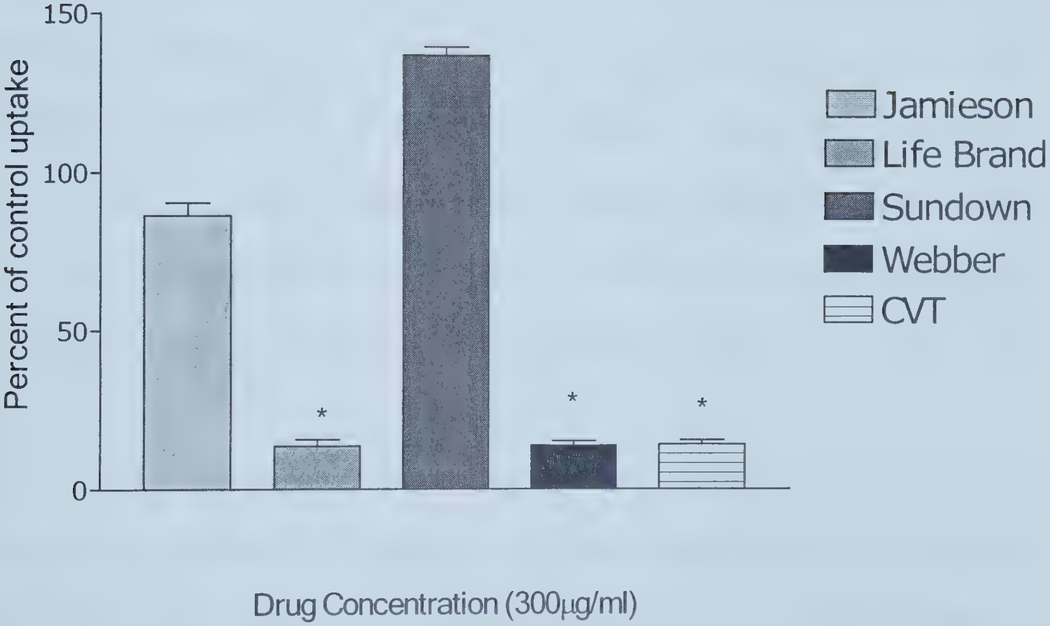


Figure 11. The effect of various commercially available preparations of SJW on ^{3}H-GABA uptake by rat cortical prisms. Results are expressed as means +/-SEM (N=4).*P<0.001.

5. DISCUSSION

5.1 Comments on the GABAse assay

5.1.1 The effects of PLZ and SJW on GABAse

GABAse is a bacterial preparation composed of two enzymes, GABA-T and SSADH. GABA-T is responsible for the breakdown of GABA to succinic semialdehyde, while SSADH is responsible for degrading succinic semialdehyde. The inhibition of GABA-T leads to increased GABA levels, and this effect may play a role in the antidepressant efficacy of some medications. An increase in GABA levels may also reduce anxiety, as common anxiolytic medications, such as the benzodiazepines, are known to increase GABAergic function in the central nervous system (Martin, 1987).

The antidepressant PLZ is not only an inhibitor of MAO but also an inhibitor of GABA-T (Baker et al., 2000) and was included as a comparator drug. AOAA was also included as a known potent GABA-T inhibitor. At the concentrations tested, SJW caused a dose-related reduction in optical density change, an indirect measure of enzyme function. However, it is not possible to conclude from these GABAse experiments whether SJW is inhibiting GABA-T activity, as the optical density change could result from inhibition of the activity of either GABA-T or SSADH (see Figure1). An HPLC assay was thus

developed, using the components of the GABAse assay, to determine which of these two enzymes were inhibited by SJW by measuring levels of glutamate. These effects are described below.

5.1.2 The effects of SJW on GLU levels, measured by HPLC

As shown in Figure 1, inhibition of GABA-T should lead to lower levels of glutamate compared to control levels, while inhibition of only SSADH should result in an increase in glutamate levels. SJW produced levels of GLU that were significantly decreased compared to controls. This effect was also observed with PLZ and AOAA, two known inhibitors of GABA-T. Based on these findings, it is likely that SJW is inhibiting GABA-T activity and not SSADH, in the GABAse assay. An inhibition of GABA-T activity may contribute to the anxiolytic properties and calming effects of the herb since PLZ and vigabatrin, another clinically available GABA-T inhibitor, have anxiolytic properties (Baker et al., 2000).

5.1.3 The effects of SJW constituents on GABAse activity

The various SJW constituents tested, hypericin, hyperforin, quercetin, kaempferol, and pseudohypericin, did not affect the activity of GABAse, measured as a change in optical density. Therefore, some

other constituent of SJW must be responsible for the GABAse inhibiting effects of SJW.

5.1.4 The effect of SJW on ^{3}H-GABA uptake

The cortical uptake of GABA *in vitro* was inhibited significantly by SJW extract in a dose-dependent manner, and this effect was more potent than the effect on inhibition of GABAse activity. In comparison with a known uptake inhibitor, nipecotic acid, these effects were relatively potent. It is conceivable that this inhibition could contribute to the antidepressant and/or antianxiety effects of SJW since numerous drugs which increase GABA availability have such actions and decreased GABA levels have been reported in plasma, CSF and brain tissue of depressed subjects (see the Introduction section of this thesis). The inhibition of GABA uptake has a number of potential effects clinically, including anxiolytic, calming, and antiepileptic effects.

A variety of SJW constituents were tested for ^{3}H-GABA uptake inhibiting efficacy but with the exception of HYPF, none was found to exhibit this effect at the concentrations tested.

HYPF was tested at various concentrations, and the inhibition of ^{3}H-GABA uptake was found to be dose-related. In the literature, there

are reports that the HYPF content of SJW extract correlates well with the clinical response. Muller et al., (2001) have shown that HYPF inhibits the uptake of 5-HT, DA, and NA as well as GABA and GLU within the same concentration range, but these researchers did not investigate the other constituents of SJW. Pharmacokinetic studies have shown that HYPF reaches high nanomolar to low micromolar concentrations in plasma (Barnes et al., 2001; Wonnemann et al., 2001). Thus, it is reasonable to predict that this constituent, at least in part through its action of GABA reuptake, may indeed play a role in the clinical efficacy of SJW.

5.1.5 The effects of various commercially available SJW extract preparations

A variety of different commercial preparations reported to have the same amount of HYPC were tested in the ^{3}H-GABA uptake assay. The inhibition observed was inconsistent, with three brands exhibiting fairly potent inhibition, while two others showed no inhibitory effects. Discrepant clinical findings among SJW clinical trials (see section 1.6), have been the subject of great debate and many clinicians and scientists are encouraging the use of stricter guidelines in the preparation and sale of health food products. At the present time, there are no guarantees that the SJW product will contain any active

ingredient. Currently SJW is standardized on HYPC content and the label on most commercial preparations indicates that the extract contains no less than 0.3% HYPC. However, a study that analyzed the HYPC content of a number of German commercial SJW preparations found that the actual HYPC content ranged from 0 to 120% of the advertised content (Wurglics et al., 2001). The range of uptake inhibition observed with the different commercial preparations tested in this thesis may be related to a range in HYPF content, but this content was not indicated on the preparations. Given these findings and reports in the literature strongly suggesting that HYPF plays an important role in the clinical efficacy of SJW (Jensen et al., 2001; Wonnemann et al., 2001), it would be useful in the future to standardize commercial SJW preparations with respect to their HYPF content.

6. SUMMARY

The effects of SJW and several of its constituents on two mechanisms important in the control of brain GABA, namely inhibition of GABA-T and inhibition of GABA uptake, were investigated. Studies on GABAse using two different techniques indicated an inhibitory effect of SJW on GABA-T. However, this effect was not produced by any of the major known constituents of SJW, indicating that another constituent is responsible for this action. An overall extract of CVT brand SJW inhibited GABA uptake into cortical prisms from rat brain. Subsequent experiments implicated a significant role for HYPF in the mechanism of action of SJW. The HYPF content of SJW is roughly 5% and HYPF concentrations corresponding to 5% of the SJW concentrations tested produced similar uptake inhibiting effects. A comparison of the effects of five commercially available brands of SJW, all standardized to the same amount of HYPC, showed that 3 preparations caused considerable inhibition of ^{3}H-GABA uptake, while two did not. These results emphasize the variability and inconsistency of activity among herbal products. Although the labels on the products claimed that the extracts were standardized, the uptake inhibiting effects were unpredictable and indicates that in future experiments on the mechanism(s) of action of SJW, levels of HYPF in the products tested should be analyzed routinely.

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